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APPLICATION NUMBER: ***60/783,070***
FILING DATE: ***March 15, 2006***
RELATED PCT APPLICATION NUMBER: ***PCT/US07/06606***

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS ***US60/783,070***

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031506
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ED 481024650 US

112987 U.S. PTO
60/783070
031506

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Leonard	Bell	Woodbridge, Connecticut
Russell P.	Rother	Prospect, Connecticut

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number of CDs _____
☑ Specification *Number of Pages* 32
☐ Other (specify) _____
☑ Drawing(s) *Number of Sheets* 4

Fees Due: Filing Fee of $200 ($100 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fee and application size fee (if applicable).
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☑ The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any overpayment to Deposit Account Number: 01-0483. A duplicative copy of this form is enclosed for fee processing.

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TOTAL FEE AMOUNT ($)

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SIGNATURE Stephen A. Saxe Date March 15, 2006

TYPED or PRINTED NAME Stephen A. Saxe REGISTRATION NO. 38,609 *(if appropriate)*

TELEPHONE 203-271-8289 Docket Number: 114 CIP2 PRO

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Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2006

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 100.00

Complete if Known	
Application Number	Not yet assigned
Filing Date	March 15, 2006
First Named Inventor	Leonard Bell
Examiner Name	Not yet assigned
Art Unit	N/A
Attorney Docket No.	114 CIP2 PRO

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☑ Deposit Account Deposit Account Number: 01-0483 Deposit Account Name: Alexion Pharmaceuticals

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

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	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	100.00

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee ($)		Fee Paid ($)
_____	- 20 or HP =	_____	x	_____	=	0

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee ($)		Fee Paid ($)
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HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	0

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
36	- 100 =	0	/ 50 =	0	(round up to a whole number) x	_____	=	0

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	0
Other (e.g., late filing surcharge): _____	0

SUBMITTED BY

Signature	Stephen A. Saxe	Registration No. (Attorney/Agent) 38,609	Telephone 203-271-8289
Name (Print/Type)	Stephen A. Saxe		Date March 15, 2006

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TRANSMITTAL FORM

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Total Number of Pages in This Submission: 41

Application Number	Not yet assigned
Filing Date	March 15, 2006
First Named Inventor	Leonard Bell
Art Unit	N/A
Examiner Name	N/A
Attorney Docket Number	114 CIP2 PRO

ENCLOSURES *(Check all that apply)*

- [x] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
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  - [ ] Affidavits/declaration(s)
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Provisional Application for Patent Cover Sheet (2 pages)
Provisional Application (36 pages)
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Remarks

Total pages (41) equals 32 pages of specification, 4 sheets of drawings, 2 pages of Provisional Application for Patent Cover Sheet, 1 page of Fee Transmittal, 1 page of Transmittal Form and 1 page of Certificate of Express Mailing. Additionally, a Return Receipt Postcard is enclosed.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Stephen A. Saxe, Alexion Pharmaceuticals, Inc.		
Signature	Stephen A. Saxe		
Printed name	Stephen A. Saxe		
Date	March 15, 2006	Reg. No.	38,609

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Docket No.: 114 CIP2 PRO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Leonard BELL and Russell P. ROTHER		
Serial No.:	Unassigned	Examiner:	Unassigned
Filed:	March 15, 2006	Art Unit:	Unassigned

For: **IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT**

CERTIFICATE OF EXPRESS MAILING

Date of Deposit: March 15, 2006
I hereby certify that the following:
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[x] This Certificate of Express Mailing
[x] Provisional Application for Patent Cover Sheet (2 pages)
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[x] Specification (32 pages)
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Stephen A. Saxe

Stephen A. Saxe
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352 Knotter Drive
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Tel. 203-271-8289

IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A.[1,2] Mutations in PIG-A lead to an early block in the synthesis of glycosylphosphatidylinositol (GPI)-anchors, which are required to tether many proteins to the cell surface. Consequently, PNH blood cells have a partial (type II) or complete (type III) deficiency of GPI-anchored proteins.

Intravascular hemolysis is a prominent feature of PNH and a direct result of the absence of the GPI-anchored complement regulatory protein CD59.[3,4] Under normal circumstances, CD59 blocks the formation of the terminal complement complex (also called the membrane attack complex) on the cell surface, thereby preventing erythrocyte lysis and platelet activation.[5-8] Excessive or persistent intravascular hemolysis in PNH patients not only results in anemia (normal ranges of hemoglobin are 14-18 g/dL for men and 12-16 g/dL for women, and persons with lower levels are considered to be anemic), but also hemoglobinuria and clinical sequelae related to the release of the erythrocyte contents into the circulation: fatigue, thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9,10,21,22] Indeed, impaired quality of life in PNH is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate erythrocyte hemoglobin levels. There have been no therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH.

Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5.[11] In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards.

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SUMMARY

The present pivotal, phase III study, Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH), evaluated the effect of eculizumab on the stabilization of hemoglobin levels and transfusion requirements during 6 months of treatment in a cohort of 87 transfusion-dependent PNH patients. Measures of intravascular hemolysis and quality of life were also assessed. This is the first placebo controlled study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effects due to anemia.

It has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab. Furthermore, these improvements in the quality of life were independent of transfusion. The improved aspects include, e.g., global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss and insomnia. Improvement was also seen in nausea and vomiting, diarrhea, constipation, and financial difficulties but did not reach the level of statistical significance. Because the treated patients remained anemic throughout their treatment, it was unexpected that all of these improvements would have been seen because they were previously thought to be a result of the patient being anemic. Although not wishing to be bound by any theory, it appears that some of the symptoms are likely due, at least in part, to hemolysis and release of hemoglobin into the bloodstream and do not result solely from the patient being anemic. The treatment with eculizumab decreases the amount of lysis thereby limiting hemoglobin release into the bloodstream, thereby apparently resulting in the improvements seen in the treated patients' quality of life. The results presented herein indicate that any treatment that decreases hemolysis in a patient will result in an improvement in the quality of life of said patient.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B show that eculizumab treatment decreases intravascular hemolysis and increases PNH type III erythrocytes. Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels.

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Figure 1B shows the mean proportion of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients.

Figure 2 shows the effect of eculizumab treatment on transfusion requirements in PNH patients. This is a Kaplan-Meier plot of time to first transfusion for eculizumab- and placebo-treated patients from baseline through week 26. The P value is from the log rank analysis.

Figure 3 shows the effect of eculizumab on fatigue assessed by the FACIT-Fatigue Instrument. Quality of Life scores were assessed using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. Values for change from baseline to 26 weeks represent least-square means. A positive change indicates an improvement and a negative change indicates deterioration in the FACIT-Fatigue measures of quality of life.

DETAILED DESCRIPTION

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH"), more specifically to improving certain aspects of quality of life which are impaired in PNH patients, and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red

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blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the present methods. A specific class of such compounds which is particularly useful includes antibodies specific to a human complement component, especially anti-C5 antibodies. The anti-C5 antibody inhibits the complement cascade and, ultimately, prevents red blood cell ("RBC") lysis by the complement protein complex C5b-9. By inhibiting and/or reducing the lysis of RBCs, the effects of PNH and other hemolytic diseases (including symptoms such as hemoglobinuria, anemia, hemoglobinemia, dysphagia, fatigue, erectile dysfunction, recurrent abdominal pain and thrombosis) are eliminated or decreased.

Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, Mass.), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

The complement system, useful complement inhibitors, and use of these inhibitors to treat PNH and other patients are more fully described in PCT Patent Application PCT/US2005/003225 filed February 3, 2005 and published as International Publication Number WO 2005/074607 A2 on August 18, 2005, the contents of which are incorporated herein by reference in their entirety.

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I. METHODS

Patient Selection

The TRIUMPH trial consisted of a 2-week screening period, an observation period of up to 3 months duration, and a 26-week treatment period.

During the screening period, patients were evaluated with respect to inclusion and exclusion criteria. Men and women, 18 years or older, diagnosed as having PNH with a type III erythrocyte population of ≥10%, and who had received at least 4 transfusions in the previous 12 months were eligible. Concomitant administration of erythropoietin, immunosuppressants, corticosteroids, coumadin, low molecular weight heparin, iron supplements, and folic acid were not reasons for exclusion, provided the doses were steady prior to the first visit and throughout the duration of the study. Because of the increased frequency of neisserial infections in individuals genetically deficient in terminal complement proteins, all patients were vaccinated against *Neisseria meningitides*. Patients were to avoid conception. The protocol was approved by an Investigational Review Board at each clinical site and written informed consent was obtained from all patients enrolled.

Patients transfused with a mean pre-transfusion hemoglobin level >10.5 g/dL over the previous 12 months, and those who showed evidence of having a suppressed immune response, complement deficiency, or active bacterial infection, including any history of meningococcal disease, were excluded from the study. Patients were also not eligible if they had previously received a bone marrow transplant or if they had participated in another trial or received another investigational drug within 30 days of the first visit. An individualized transfusion algorithm was calculated for each patient based on their prior 12-month transfusion history; the written algorithm documented the number of packed red blood cell (PRBC) units transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during observation and treatment periods.

Each patient considered eligible entered an observation period of up to 13 weeks in order to confirm their PBRC transfusion dependence. At least one transfusion --termed the "qualifying" transfusion-- during the 13-week observation period at a hemoglobin value at or below 9 g/dL with symptoms, or at or below 7 g/dL with or without symptoms, in accordance with the transfusion algorithm indicated for each patient, was a

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requirement to proceed to randomization. The hemoglobin value at which each individual's qualifying transfusion was administered, was defined as the hemoglobin "set point" for that individual for the purpose of the primary efficacy variable. A platelet count ≥100,000/mL and a LDH level ≥1.5 times the upper limit of the normal range were also required either at screening or during the observation period for eligibility.

Study Design

Patients were randomly assigned on a one-on-one basis to receive either placebo or eculizumab (Soliris™, Alexion Pharmaceuticals, Inc.) within 10 days of the qualifying transfusion. Study medication was dosed in a blinded fashion as follows: 600 mg eculizumab for patients randomly assigned to active drug, or placebo for those patients randomly assigned to placebo, respectively via IV infusion every 7±1 days for 4 doses; followed by 900 mg eculizumab, or placebo, respectively, via IV infusion 7±1 day later; followed by a maintenance dose of 900 mg eculizumab, or placebo, respectively, via IV infusion every 14±2 days for a total of 26 weeks of treatment.

Measures of Clinical Efficacy

There were two co-primary endpoints in the study: (1) stabilization of hemoglobin levels, defined as a hemoglobin value maintained above the individual hemoglobin set point in the absence of transfusions for the entire 26-week treatment period, and (2) reduction in units of PRBCs transfused during the 26-week treatment phase of the study. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia and reached their individualized, pre-determined "set point". Pre-specified secondary endpoints included transfusion avoidance, hemolysis as measured by LDH area under the curve from baseline to 26 weeks, and QoL changes as measured from baseline to 26 weeks using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument.[13] Pre-specified exploratory analyses included assessment of the EORTC QLQ-C30 instrument,[14] the change in LDH from baseline through week 26, and thrombosis. Other pre-specified measurements included pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. Time to first transfusion during

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the 26-week treatment phase and the proportion of PNH type III blood cells were also assessed.

Safety Assessments

Treatment-emergent adverse events, clinical laboratory tests (e.g., serum chemical analyses and complete blood counts), electrocardiogram data, and vital signs were assessed. Adverse events were defined using the MedDRA preferred terms and tabulated as incidence rates per treatment group.

Statistical Analysis

For co-primary endpoints, analyses were performed according to the intention to treat using the data from all patients who were randomized and received study drug; stabilization of hemoglobin levels was analyzed using the Fisher's exact test and total PRBC units transfused were analyzed with the Wilcoxon's rank sum test. For comparison of treatment effect on transfusion avoidance, the Fisher's exact test was used on the incidence and the log rank test was used for time to first transfusion. For LDH area under the curve the Wilcoxon's rank sum test was used.

Quality of life measure of fatigue was assessed using the scoring guidelines for the FACIT-Fatigue instrument.[15] Assessment of quality of life measures based on the EORTC QLQ-C30 instrument was conducted in accordance with the appropriate scoring guidelines.[16] The changes of FACIT-Fatigue and EORTC QLQ-C30 scores from baseline through 26 weeks were analyzed using a mixed model, with baseline as covariate, treatment and time as fixed effects, and patient as a random effect. Changes in LDH levels and PNH type III erythrocytes from baseline through 26 weeks were analyzed using the same mixed model. Two-sided tests were used for all analyses. The adverse events and long-term safety checklist were tabulated separately and compared between treatments using the Fisher's exact test. A p-value ≤ 0.05 was considered to be statistically significant.

II. RESULTS

Characteristics of Patients

A total of 115 PNH patients were screened. Six patients did not meet the inclusion/exclusion criteria during the screening period. Twenty-one other patients did

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not receive a qualifying transfusion and were not randomized into the treatment phase. One patient who did not meet the inclusion criteria was inadvertently randomized, but did not receive study medication. Thus 87 hemolytic PNH patients (35 men and 52 women) were enrolled and randomized to receive either eculizumab (N=43) or placebo (N=44), exceeding the original target of 75 randomized patients.

Patient characteristics were similar in the eculizumab- and placebo-treated cohorts: median age, 41 (range 20-85) and 35 (range 18-78) years; median duration of PNH, 4.2 (range 0.8 to 29.7) and 9.2 (range 0.4 to 38.3) years; patients with history of aplastic anemia, 4 and 11; history of myelodysplastic syndrome, 1 and 0; and history of thrombosis, 9 (16 events) and 8 (11 events). Stable usage of concomitant medications at baseline in the eculizumab- and placebo-treated groups included the following: erythropoietin, 3 patients and 0 patients; cyclosporine, 1 and 1; anticoagulants (coumarins or heparins) 21 and 11; and steroids (glucocorticoids or androgenic steroids), 12 and 12, respectively.

Of the 87 patients randomized, 85 completed the trial. Two patients who did not complete the trial had been randomized to the eculizumab arm: one patient discontinued due to the inconvenience of travel to the study site and the second patient became pregnant. Ten patients in the placebo-treatment group discontinued infusions, in all cases due to perceived lack of efficacy, but they remained in the study for monitoring purposes.

Pharmacokinetics/Pharmacodynamics

In 42 of the 43 eculizumab-treated patients the levels of drug during the maintenance period (900 mg every 2 weeks±2 days) were sufficient to completely block serum hemolytic activity (mean trough value at week 26 of 101.8 μg/mL). A single patient did not sustain therapeutic trough levels of eculizumab and demonstrated a breakthrough in complement blockade during the last few days of each dosing interval. These breakthroughs were clinically manageable and quickly resolved following the next dose.

Hemolytic Efficacy Variables

The impact of terminal complement inhibition with eculizumab on chronic intravascular hemolysis in PNH patients was demonstrated in this study by an immediate (one week) and sustained decrease in mean levels of LDH (Figure 1A). The median LDH

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area under the curve during the 26-week study period was reduced 85.8% in eculizumab- relative to placebo-treated patients (P<0.001). The mean LDH level decreased from 2199.7±157.7 IU/L at baseline to 327.3±67.6 IU/L by 26 weeks in eculizumab-treated patients while levels in placebo-treated patients remained consistently elevated with values of 2259.0±158.5 IU/L at baseline and 2418.9±140.3 IU/L at 26 weeks (P<0.001, for eculizumab versus placebo). A second biochemical measure of hemolysis, serum aspartate aminotransferase (AST), also showed a statistically significant improvement following eculizumab- versus placebo-treatment (data not shown). Haptoglobin levels were statistically significantly increased in eculizumab- as compared to placebo-treated patients but mean levels of haptoglobin were still below normal levels in eculizumab-treated patients (data not shown).

Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels (±standard error) from baseline (study week 0) to week 26 for both eculizumab- and placebo-treated patient populations. Screening occurred up to 3 months prior to study week 0. The upper limit of the normal range (103-223 IU/L) for LDH is indicated by a dashed line. LDH was reduced to a mean level just above the upper limit of normal at week 26 for eculizumab-treated patients; 15 of 41 patients who completed the study demonstrated LDH levels within the normal range. All placebo-treated patients remained at least 5 times above the upper limit of normal at week 26. The P value is based on a mixed model analysis from baseline through week 26. Figure 1B shows the mean proportion (±standard error) of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients. The screening visit occurred up to 3 months prior to study week 0. The P value is based on a mixed model analysis from baseline through week 26.

A corollary to the reduction in intravascular hemolysis during eculizumab treatment was an observed increase in the PNH type III erythrocyte population (Figure 1B). The mean proportions of type III erythrocytes increased from 28.1±2.0% at baseline to 56.9±3.6% by week 26 for eculizumab-treated patients while proportions in the placebo group remained constant with mean values of 35.7±2.8% before treatment to 35.5±2.8% at 26 weeks (P<0.001, for eculizumab versus placebo). By contrast, the proportions of PNH type III granulocytes and monocytes did not change significantly

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between the treatment groups during the treatment period and were greater than 90% at week 26.

III. CLINICAL EFFICACY

Co-primary endpoints

The co-primary efficacy endpoints in the TRIUMPH trial were stabilization of hemoglobin levels and reduction in PRBC units transfused. At the end of the treatment period, 48.8% of eculizumab-treated patients had maintained levels of hemoglobin above the pre-specified set-point (median set-point value of 7.7 g/dL for both treatment groups) in the absence of transfusions, whereas stabilization of hemoglobin did not occur in any of the patients in the placebo group ($P<0.001$; Table 1). By week 26, the median of PRBC units transfused per patient was 0 in the eculizumab group and 10.0 in the placebo cohort ($P<0.001$), while the mean of PRBC units transfused was 3.0 and 11.0 in the eculizumab and placebo cohorts, respectively. In the 6-month period prior to the study, the median of PRBC units transfused per patient was 9.0 in the eculizumab cohort and 8.5 in placebo patients while the mean of PRBC units transfused was 9.6±0.6 and 9.7±0.7, respectively. Mean hemoglobin levels were similar between the treatment groups at baseline (10.0±1.8 g/dL in eculizumab-treated patients and 9.7±1.8 g/dL in placebo-treated patients) and did not substantially change by week 26 (10.1±2.5 g/dL and 8.9±2.2 g/dL in eculizumab and placebo cohorts, respectively).

The median time to first transfusion was not reached during the study period in eculizumab-treated patients (it was greater than 26 weeks) while the placebo group reached the median time to first transfusion in only 4 weeks ($P<0.001$; Figure 2). Transfusion avoidance was achieved in 51.2% and 0% of the eculizumab and placebo cohorts, respectively ($P<0.001$). By the end of the 26-week treatment period, the total PRBC units transfused were 131 in eculizumab-treated patients versus 482 in the placebo group (Table 1). By contrast, in the 6-month period prior to the study, total PRBC units transfused in the eculizumab- and placebo-cohorts were 413 and 417, respectively.

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

	PRE-TREATMENT HISTORY*		TREATMENT PERIOD		
	Placebo	Eculizumab	Placebo	Eculizumab	P Value
†Stabilization of Hemoglobin Levels in the absence of transfusions (percent of patients)	NA	NA	0	48.8	<0.001‡
†Units Transfused per Patient					
Median	8.5	9.0	10	0	<0.001§
Mean±SE	9.7±0.7	9.6±0.6	11.0±0.83	3.0±0.67	
Total Units Transfused per Group	417	413	482	131	

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

Assessments of quality of life in PNH patients during eculizumab treatment were performed using two different instruments, FACIT-Fatigue and the EORTC QLQ-C30. Eculizumab-treated patients showed a mean increase (improvement) in the FACIT-Fatigue score of 6.4±1.2 points from baseline to 26-weeks while the mean score in placebo patients decreased by 4.0±1.7 points, a total difference between the treatment groups of 10.4 points (Figure 3). Mixed model analysis of covariance demonstrated a statistically significant difference between treatment groups (P<0.001).

For the EORTC instrument, improvements were observed with eculizumab-treatment in each subscale. Statistically significant improvements with eculizumab-

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compared with placebo-treated groups were observed in the following quality of life subscales (Table 2): global health status (P<0.001), physical functioning (P<0.001), emotional functioning (P=0.008), cognitive functioning (P=0.002), role functioning (P<0.001), social functioning (P=0.003), fatigue (P<0.001), pain (P=0.002), dyspnea (P<0.001), appetite loss (P<0.001), and insomnia (P=0.014). The improvements with eculizumab treatment in the other scales, including nausea and vomiting, diarrhea, constipation, and financial difficulties, did not reach statistical significance.

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

	Mean Change from Baseline to Week 26*			
	Placebo	**Eculizumab**	**Absolute Difference**	**P Value†**
Global Health Status	-8.5	10.9	19.4	<0.001
Functional				
Role	-6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.6	=0.003
Cognitive	-6.1	7.9	14.0	=0.002
Physical	-3.5	9.4	13.0	<0.001
Emotional	-3.7	7.5	11.2	=0.008
Symptoms/Single Items				
Fatigue	10.0	-16.9	27.0	<0.001
Pain	5.3	-12.3	17.6	=0.002
Dyspnea	8.9	-7.9	16.9	<0.001
Appetite Loss	3.3	-10.3	13.6	<0.001
Insomnia	4.9	-7.9	12.8	=0.014

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Financial Difficulties	0.0	-10.3	10.3	=0.186
Constipation	0.0	-6.3	6.3	=0.199
Nausea/vomiting	2.8	-0.4	3.2	=0.056
Diarrhea	5.7	4.8	0.9	=0.147

*A positive change indicates an improvement in global health status and functional scales and a negative change indicates an improvement in symptom and single item scales.

†Based on a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect and baseline as a covariate.

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

In order to determine if there was a treatment independent relationship between the FACIT-Fatigue quality of life instrument and intravascular hemolysis, an analysis was performed whereby the mean LDH level (through the 26 week study period) for each TRIUMPH patient was analyzed as a function of the patient's respective mean change in FACIT-Fatigue score from baseline (through the 26 week study period) (see Table 3). For this analysis, mean levels of LDH were divided into 4 groups that included: normal levels, 1-2 times the upper limit of normal (ULN), 2-10 times the upper limit of normal, and greater than 10 times the upper limit of normal. The analysis demonstrated that patients who maintained normal LDH levels throughout the study experienced significant improvements in fatigue when compared to patients who had incrementally higher levels of LDH throughout the study ($p=0.0048$). These data establish a clear link between increasing intravascular hemolysis as measured by LDH levels and decreased quality of life as measured by the FACIT-Fatigue quality of life instrument.

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

		Change of FACIT from Baseline		
Treatment Group	LDH Category	< 4	>= 4	P Value
Combined	Normal	4 (30.77%)	9 (69.23%)	.0048
	1 - 2 X ULN	16 (61.54%)	10 (38.46%)	
	2 - 10 X ULN	18 (72.00%)	7 (28.00%)	
	> 10 X ULN	16 (80.00%)	4 (20.00%)	

Safety

There were no deaths in the study. Serious adverse events (SAEs) were reported for 13 patients, of which 4 occurred in the eculizumab-treated cohort and 9 were in the placebo-treated cohort (see Table 4). All patients recovered without sequelae.

The most common AEs reported for eculizumab-treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infection. Headache and back pain occurred more commonly in the eculizumab-treatment group compared with the placebo group. However, the increase in headaches was limited to the first 2 weeks of therapy and was mild to moderate. There were no statistically significant differences in incidents rates between treatment groups for any AEs reported.

One episode of thrombosis (Budd-Chiari) occurred in a placebo-treated patient. There were no thromboses in eculizumab-treated patients.

Only one patient showed a detectable level of anti-eculizumab antibodies in the eculizumab-treated cohort; this response was weak (did not titrate), occurred at only one time point and did not result in a disruption of complement blockade.

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Table 4

Adverse Event Reporting

SERIOUS ADVERSE EVENTS*	**Placebo Patients n (percent)**	**Eculizumab Patients n (percent)**
Total	9 (20.5)	4 (9.3)
Eculizumab treatment emergent		
Exacerbation of PNH	3 (6.8)	1 (2.3)
Renal colic	0	1 (2.3)
Lumbar sacral disc prolapse	0	1 (2.3)
Alpha streptococcal bacteremia	0	1 (2.3)
Central line infection and UTI	1 (2.3)	0 (0)
Upper respiratory tract infection	1 (2.3)	0 (0)
Probable viral infection	1 (2.3)	0 (0)
Neutropenia	1 (2.3)	0 (0)
Cellulitis / folliculitis / neutropenia	1 (2.3)	0 (0)
Anemia and pyrexia	1 (2.3)	0 (0)
MOST FREQUENT ADVERSE EVENTS*†		
Headache§	12 (27.3)	19‡ (44.2)
Nasopharyngitis	8 (18.2)	10 (23.3)
Upper respiratory tract infection	10 (22.7)	6 (14)
Back pain	4 (9.1)	8 (18.6)
Nausea	5 (11.4)	7 (16.3)
Cough	4 (9.1)	5 (11.6)
Diarrhea	5 (11.4)	4 (9.3)
Arthralgia	5 (11.4)	3 (7.0)
Abdominal pain	5 (11.4)	2 (4.7)
Dizziness	5 (11.4)	2 (4.7)
Vomiting	5 (11.4)	2 (4.7)

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Fatigue	1 (2.3)	5 (11.6)
Viral infection	5 (11.4)	1 (2.3)

*By preferred terms

†Occurring in 10% or more of patients

‡Sixteen of 19 patients experienced headache within 48 hours of infusion

§Following the first 2 weeks of dosing, 20.9% of eculizumab- and 22.7% of placebo-treated patients experienced headache

IV. DISCUSSION

Chronic intravascular hemolysis with periods of acute exacerbation are the classical manifestations of PNH, frequently resulting in anemia, the need for transfusions to sustain hemoglobin levels, and deterioration in quality of life. In the phase III pivotal TRIUMPH study, we examined the effect of terminal complement inhibition with eculizumab on hemoglobin levels and transfusion requirements in patients with PNH. Forty-nine percent of patients treated with eculizumab over the 6-month period demonstrated stabilization of hemoglobin in the absence of transfusions compared to no patients in the placebo arm of the trial. Over 50% of eculizumab-treated patients were transfusion independent during the entire study compared to none in the placebo arm, and the overall mean transfusion rate was reduced by 73%. Moreover, even in patients who did not achieve transfusion independence, eculizumab treatment was associated with a 44% reduction in the rate of transfusion (data now shown).

Lactate dehydrogenase, a biochemical marker of hemolysis in PNH,[9] was immediately and consistently decreased in all eculizumab-treated patients, while patients in the placebo cohort continued to hemolyze with levels of LDH exceeding 5 times the upper limit of the normal range in all patients at the study end. Levels of LDH were reduced into the normal range in approximately one-third of eculizumab-treated patients, while the remainder stabilized at a level just above the upper limit of normal suggesting residual low level hemolysis in some patients. Levels of haptoglobin, a more sensitive marker of the presence of cell free hemoglobin in the circulation, were undetectable in most patients. Low level hemolysis in a subset of eculizumab-treated patients is possibly

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due to an inherent decrease in survival of these cells or C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.[17]

Before eculizumab treatment, hemoglobin levels in study patients were artificially maintained by frequent transfusion. Therefore, stabilization of hemoglobin levels with a concomitant cessation of or reduction in transfusions represents a net increase in endogenous hemoglobin levels. Our data suggest that resolution of hemolysis with eculizumab results in a new steady state hemoglobin level determined by a balance between the extent of the underlying bone marrow dysfunction, the number of PNH erythrocytes that are preserved by eculizumab therapy and the new level (if any) of transfusion requirement.

Patients with PNH generally experience markedly impaired quality of life characterized by fatigue, anemia, thrombosis, and pulmonary hypertension as well as smooth muscle dystonia including abdominal pain, dysphagia, and erectile dysfunction.[9,10,18] These symptoms have been attributed to both excessive intravascular hemolysis and downstream scavenging of nitric oxide by cell free hemoglobin in plasma. The reduction of intravascular hemolysis in eculizumab-treated patients in the current study was associated with significant improvements in the fatigue component of quality of life relative to placebo-treated patients as assessed via the FACIT-Fatigue instrument. Further, eculizumab therapy was associated with a median increase of 6.4 points over baseline values established before treatment. It has previously been demonstrated that an increase of 3 or more points from baseline represents a clinically important difference in this quality of life instrument.[19] Patients who received eculizumab also experienced a significant improvement in most domains of the EORTC QLQ-30 relative to the placebo-treated cohort including global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss, and insomnia. Improvement in the fatigue component of the EORTC QLQ-30 provides support for the improvement demonstrated in the FACIT-Fatigue instrument during eculizumab therapy. Importantly, these improvements in quality of life in the eculizumab-treated patients occurred despite similar levels of erythrocyte hemoglobin in the two treatment groups, further supporting the contribution of hemolysis per se, as opposed to anemia, in mediating the poor quality of life in PNH patients. Clinical

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assessment of additional life quality-related symptoms of PNH, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[20]

Eculizumab was safe and well-tolerated. There were no deaths in the study and only a single thrombotic event which occurred in a placebo patient in a site (the hepatic veins) which is typical of the thrombosis in PNH. The relative brief duration of this study was not sufficient to address the relevant issue of a possible protection from thrombosis by terminal complement inhibition with eculizumab.

Adverse events were generally mild with headache occurring at increased frequency in the eculizumab-treated patients; however, this increased frequency did not persist following the first two doses of therapy. There were 4 SAEs in the eculizumab treatment group and 9 SAEs in the placebo group. There was no evidence of increased infection risk in eculizumab-treated patients during the study period. One eculizumab-treated patient showed a low level of anti-eculizumab antibodies at one time point during the study which did not persist and did not result in a disruption of complement blockade. There were no AEs associated with eculizumab withdrawal in the 2 eculizumab-treated patients who did not complete the trial. Additional safety assessments, as well as efficacy measures, are being examined in an ongoing multi-center, open-label Phase III safety trial of eculizumab (SHEPHERD) in approximately 95 patients with PNH.

Results from the current randomized, double-blind, placebo-controlled, global study show that terminal complement inhibition with eculizumab appears to be a safe and effective therapy for patients with the rare disorder PNH. Treatment with eculizumab reduced intravascular hemolysis, and stabilized hemoglobin levels despite a reduction of transfusions, to the point where most PNH patients were rendered transfusion independent. Substantial and clinically meaningful improvements in fatigue and other key quality of life parameters were also demonstrated. All of the 85 patients who completed the study elected to receive eculizumab in an open-label extension study and all currently remain on drug. The results of the TRIUMPH study indicate that terminal complement inhibition with eculizumab safely and effectively addresses an important consequence of the underlying genetic defect in PNH hematopoietic stem cells by providing a therapeutic replacement for the terminal complement inhibitor deficiency.

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The present invention provides among other things treatment with an inhibitor of complement. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

All publications and patents mentioned herein, including those items listed below, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

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WHAT IS CLAIMED IS:

1. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits complement or inhibits formation of C5b-9.

2. The method of claim 1 wherein said quality of life is measured by a FACIT-Fatigue score.

3. The method of claim 2 wherein the FACIT-Fatigue score increases by at least 3 points.

4. The method of claim 2 wherein the FACIT-Fatigue score increases by ≥ 4 points.

5. The method of claim 1 wherein said quality of life is measured by an EORTC QLQ-C30 score.

6. The method of claim 5 wherein said EORTC QLQ-C30 score improves by $\geq 10\%$ of the pretreatment score.

7. The method of claim 5 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

8. The method of claim 7 wherein said aspect of quality of life is fatigue.

9. The method of claim 1 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

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10. The method of claim 1 wherein said compound is a steroid that suppresses complement.

11. The method of claim 1 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

12. The method of claim 11 wherein said compound is an antibody or an active antibody fragment.

13. The method of claim 12 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

14. The method of claim 12 wherein said antibody or active antibody fragment blocks C5 cleavage.

15. The method of claim 12 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

16. The method of claim 12 wherein said antibody is eculizumab.

17. The method of claim 12 wherein said antibody or active antibody fragment is administered for at least 6 months.

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18. The method of claim 1 wherein said patient has aplastic anemia or myelodysplastic syndrome.

19. A method to improve at least one aspect of the quality of life of an anemic patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits complement or inhibits formation of C5b-9, wherein said patient remains anemic.

20. The method of claim 19 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

21. The method of claim 19 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

22. The method of claim 19 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

23. The method of claim 19 wherein said quality of life is measured by a FACIT-Fatigue score.

24. The method of claim 23 wherein the FACIT-Fatigue score increases by at least 3 points.

25. The method of claim 23 wherein the FACIT-Fatigue score increases by ≥ 4 points.

26. The method of claim 19 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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27. The method of claim 26 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

28. The method of claim 26 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

29. The method of claim 28 wherein said aspect of quality of life is fatigue.

30. The method of claim 19 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

31. The method of claim 19 wherein said compound is a steroid that suppresses complement.

32. The method of claim 19 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

33. The method of claim 32 wherein said compound is an antibody or an active antibody fragment.

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34. The method of claim 33 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2s$, i) Fvs, j) diabodies, and k) human antibodies.

35. The method of claim 33 wherein said antibody or active antibody fragment blocks C5 cleavage.

36. The method of claim 33 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

37. The method of claim 33 wherein said antibody is eculizumab.

38. The method of claim 33 wherein said antibody or active antibody fragment is administered for at least 6 months.

39. The method of claim 19 wherein said patient has aplastic anemia or myelodysplastic syndrome.

40. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits intravascular hemolysis.

41. The method of claim 40 wherein said method results in a greater than 30% reduction in LDH in said patient.

42. The method of claim 40 wherein said quality of life is measured by a FACIT-Fatigue score.

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43. The method of claim 42 wherein the FACIT-Fatigue score increases by at least 3 points.

44. The method of claim 42 wherein the FACIT-Fatigue score increases by ≥ 4 points.

45. The method of claim 40 wherein said quality of life is measured by an EORTC QLQ-C30 score.

46. The method of claim 45 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

47. The method of claim 45 wherein said aspect of the quality of life as measured by the EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

48. The method of claim 47 wherein said aspect of quality of life is fatigue.

49. The method of claim 40 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

50. The method of claim 40 wherein said compound is a steroid that suppresses complement.

51. The method of claim 40 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA

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aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

52. The method of claim 51 wherein said compound is an antibody or an active antibody fragment.

53. The method of claim 52 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2s$, i) Fvs, j) diabodies, and k) human antibodies.

54. The method of claim 52 wherein said antibody or active antibody fragment blocks C5 cleavage.

55. The method of claim 52 wherein said antibody or active antibody fragment is administered for at least 6 months.

56. The method of claim 40 wherein said compound inhibits complement or inhibits the formation of C5b-9.

57. The method of claim 56 wherein said compound is an antibody or active antibody fragment.

58. The method of claim 57 wherein said antibody is eculizumab.

59. The method of claim 40 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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60. A method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient a compound which inhibits intravascular hemolysis, wherein said patient remains anemic.

61. The method of claim 60 wherein said method results in a greater than 30% reduction in LDH in said patient.

62. The method of claim 60 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

63. The method of claim 60 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

64. The method of claim 60 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

65. The method of claim 60 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

66. The method of claim 60 wherein said quality of life is measured by a FACIT-Fatigue score.

67. The method of claim 66 wherein the FACIT-Fatigue score increases by at least 3 points.

68. The method of claim 66 wherein the FACIT-Fatigue score increases by ≥ 4 points.

69. The method of claim 60 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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70. The method of claim 69 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

71. The method of claim 69 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

72. The method of claim 71 wherein said aspect of quality of life is fatigue.

73. The method of claim 60 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

74. The method of claim 60 wherein said compound is a steroid that suppresses complement.

75. The method of claim 60 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

76. The method of claim 75 wherein said compound is an antibody or an active antibody fragment.

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77. The method of claim 76 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2s$, i) Fvs, j) diabodies, and k) human antibodies.

78. The method of claim 76 wherein said antibody or active antibody fragment blocks C5 cleavage.

79. The method of claim 76 wherein said antibody or active antibody fragment is administered for at least 6 months.

80. The method of claim 60 wherein said compound inhibits complement or inhibits the formation of C5b-9.

81. The method of claim 80 wherein said compound is an antibody or an active antibody fragment.

82. The method of claim 81 wherein said antibody is eculizumab.

83. The method of claim 60 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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ABSTRACT

Eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation, showed activity in a preliminary 12-week open-label trial in a small cohort of patients with paroxysmal nocturnal hemoglobinuria (PNH). The present study examined whether chronic eculizumab therapy could reduce intravascular hemolysis, stabilize hemoglobin levels, reduce transfusion requirements, and improve quality of life in a double-blind, randomized, placebo-controlled, multi-center global Phase III trial. It has been found that eculizumab stabilized hemoglobin levels, decreased the need for transfusions,. and improved quality of life in PNH patients via reduced intravascular hemolysis. Chronic eculizumab treatment appears to be a safe and effective therapy for PNH.

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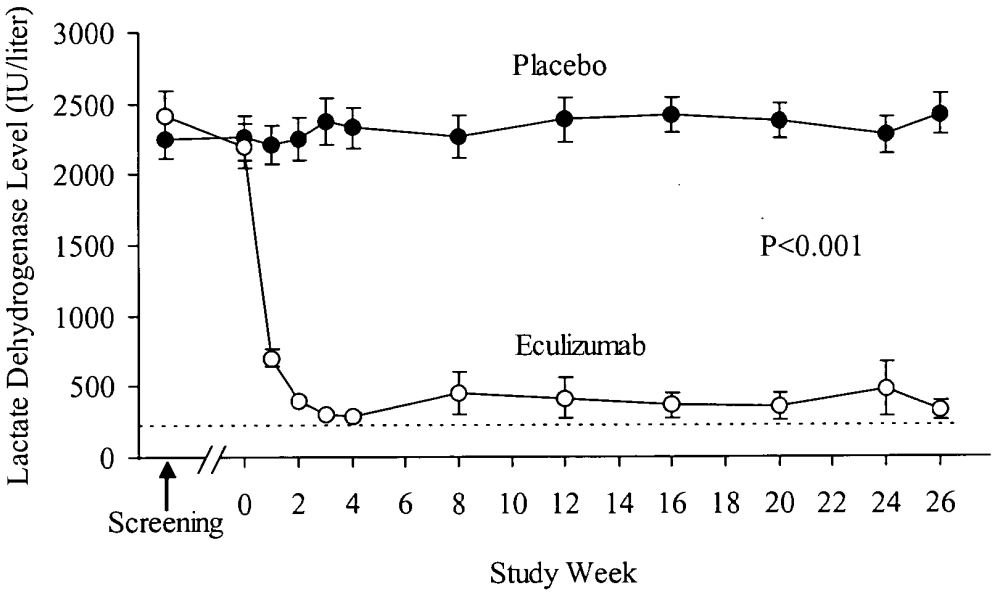

Lactate Dehydrogenase Level (IU/liter)
3000
2500
2000
1500
1000
500
0
Placebo
P<0.001
Eculizumab
Screening
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Study Week

FIGURE 1A

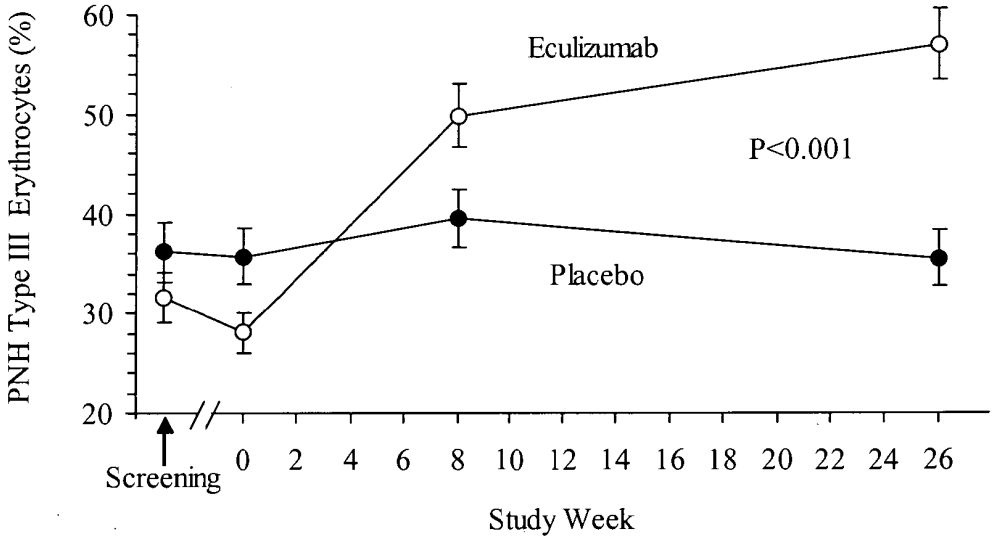

PNH Type III Erythrocytes (%)
60
50
40
30
20
Eculizumab
P<0.001
Placebo
Screening
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 1B

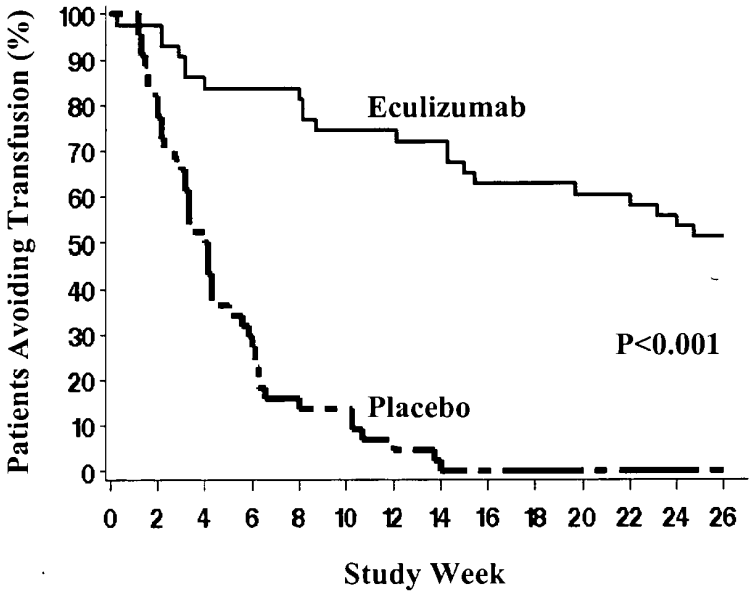

Patients Avoiding Transfusion (%)
100
90
80
70
60
50
40
30
20
10
0
Eculizumab
P<0.001
Placebo
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 2

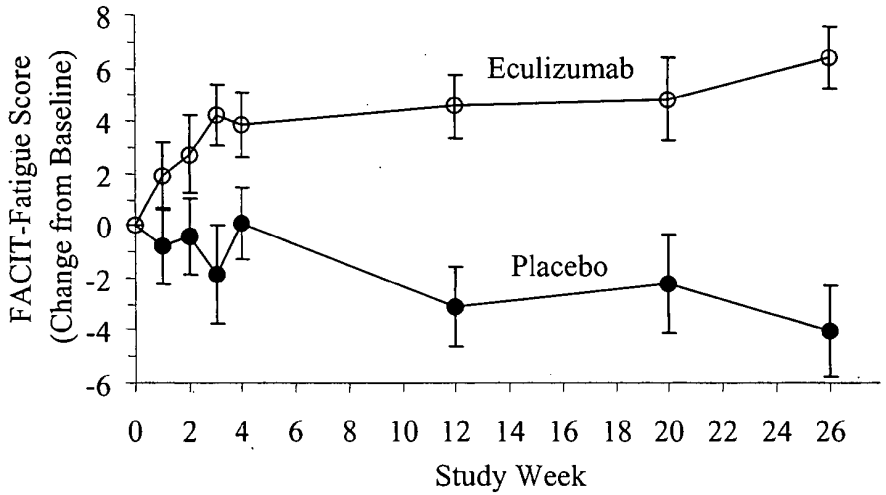


FIGURE 3

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Europäisches Patentamt
European Patent Office
Office européen des brevets

Request for grant of a European patent

For official use only		
1 Application number:	MKEY	**MKEY - EP16195355.9**
2 Date of receipt (Rule 35(2) EPC):	DREC	**DREC - 24 Oct 2016**
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

6 Applicant's or representative's reference: P2913 EP/3

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14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☐

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Inventor(s)

23 Inventor details filed separately ☒

24 **Title of invention**

Title of invention: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

25 **Declaration of priority (Rule 52)**

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	US	15.03.2006	pr	60/783,070	☐

25.2 This application is a complete translation of the previous application ☐

25.3 It is not intended to file a (further) declaration of priority ☐

26 **Reference to a previously filed application**

27 **Divisional application** ☒

Application number of earlier application: EP11001632.6

Date of Filing (Art. 80/Rule 40 EPC): 15 March 2007

27.1 This divisional application is of the following generation:

☐ 1 ☒ 2 ☐ 3

☐ 4 ☐ 5 or subsequent

28 **Article 61(1)(b) application** ☐

29 **Claims**

Number of claims: 3

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 **Figures**

It is proposed that the abstract be published together with figure No.

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Page 045

31 Designation of contracting states

All the contracting states party to the EPC and valid for the parent application at the time of filing of this divisional application are deemed to be designated (see Article 76(2)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s): BA AL HR MK RS

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description ☒

The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 ☒

The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested ☒

Application or publication number of earlier search report: EP11001632.6

42 Payment

Mode of payment: Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated on the fees section below.

Currency: EUR

Deposit account number: 28000321

Account holder: Vossius & Partner

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Page 046

Deferred execution date / total amount to be debited on: (or on the date of receipt if the submission date is after the deferred execution date you have indicated)	24.11.2016

Important information:
• This functionality allows you to indicate explicitly that your payment order is to be executed at a later date than the submission date. In this case, the payment date will be deemed to be the deferred execution date, provided that you have sufficient funds on your deposit account on that date.
• Please choose the deferred execution date option only if the fees are to be debited on a future date, as otherwise the default execution date is date of receipt.
• The deferred execution date can be a maximum of 40 days in the future.
• Please ensure that the deferred execution date is within the time limit set for paying the selected fees.
• Please note that any debit orders (standard or deferred execution) can only be revoked until 00.00 hrs CET on the date of submission (Arrangements for deposit accounts, 7)

43 Refunds

Fees	Factor applied	Fee schedule	Amount to be paid
001 Filing fee - EP direct - online	1	120.00	120.00
002 Fee for a European search - Applications filed on/after 01.07.2005	1	1 300.00	1 300.00
015 Claims fee - For the 16th to the 50th claim	0	235.00	0.00
015e Claims fee - For the 51st and each subsequent claim	0	585.00	0.00
501 Additional filing fee for the 36th and each subsequent page	26	15.00	390.00
552 Additional fee for divisional 2nd generation	1	210.00	210.00
Total:		EUR	2 020.00

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	P2913 EP_3 Specification.pdf Description; 3 claims; 3 figure(s); abstract	SPECEPO-1.pdf
B-2	Sequence listings, ASCII	P2913 EP_3 Sequence listing.txt	SEQLTXT.txt

44-C Other documents		Original file name:	System file name:
C-1	Additional sheet	P2913 EP_3 Additional sheet.pdf	OTHER-1.pdf

45	General authorisation:	

Samsung Bioepis Exhibit No. 1028
Page 047

46 Signature(s)

Place:	**München**
Date:	**24 October 2016**
Signed by:	**/Dr. Joachim Wachenfeld/**
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Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1028
Page 048

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Patentanwälte Rechtsanwälte mbB

VOSSIUS & PARTNER · POB 86 07 67 · 81634 München · Germany

European Patent Office

80298 M U N I C H

New EP divisional application
based on EP 11 00 1632.6
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

München, October 24, 2016
WA/FLG

ADDITIONAL SHEET

This is to indicate support for the claims of the above-referenced new divisional application in the parent application as filed (i.e. EP 11 00 1632.6).

1. Claims 1 and 2 are directed to an antibody that binds C5. The antibody of claim 2 comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain CDRs of SEQ ID NOs 8, 9 and 10. The antibody of claim 2 comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4. The antibodies of claims 1 and 2 have a basis at page 5, last paragraph of the parent application as filed.

2. Claim 3 is directed to a pharmaceutical composition comprising the antibody as defined in claim 1 or 2. This pharmaceutical composition has a basis at page 3, lines 18-19 in conjunction with page 5, lines 30-33 of the parent application as filed.

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4051 Basel/Switzerland
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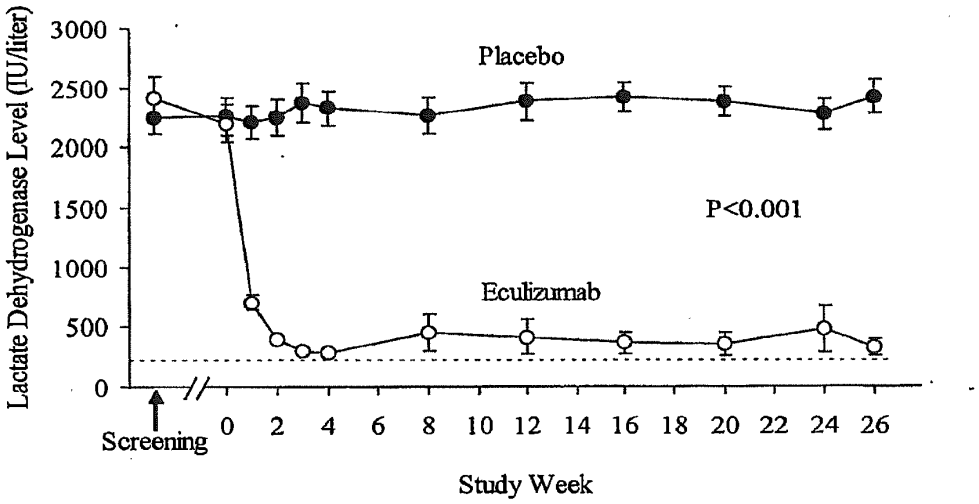

Lactate Dehydrogenase Level (IU/liter)
3000
2500
2000
1500
1000
500
0
Placebo
P<0.001
Eculizumab
Screening
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 1A

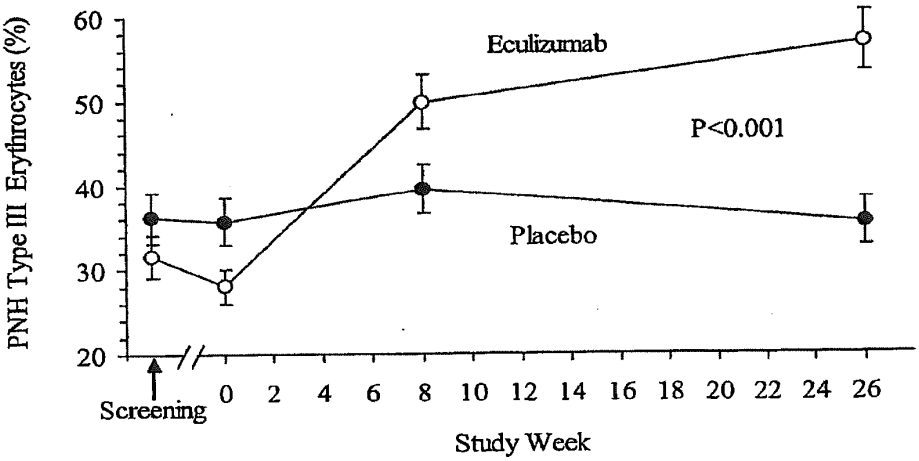

PNH Type III Erythrocytes (%)
60
50
40
30
20
Eculizumab
P<0.001
Placebo
Screening
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 1B

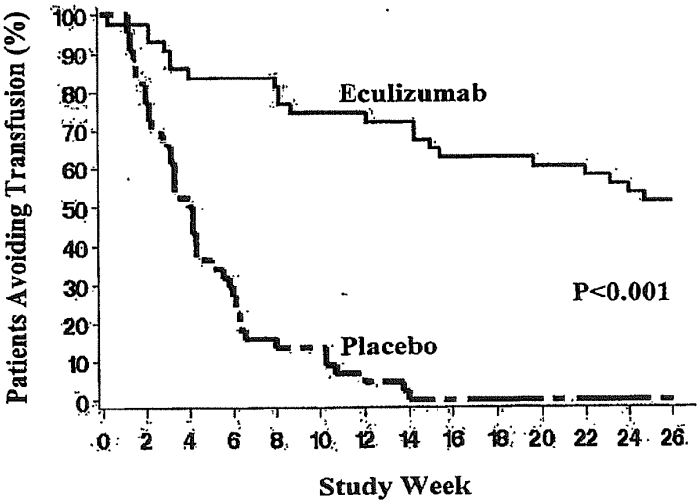

Patients Avoiding Transfusion (%)
100
90
80
70
60
50
40
30
20
10
0
Eculizumab
P<0.001
Placebo
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 2

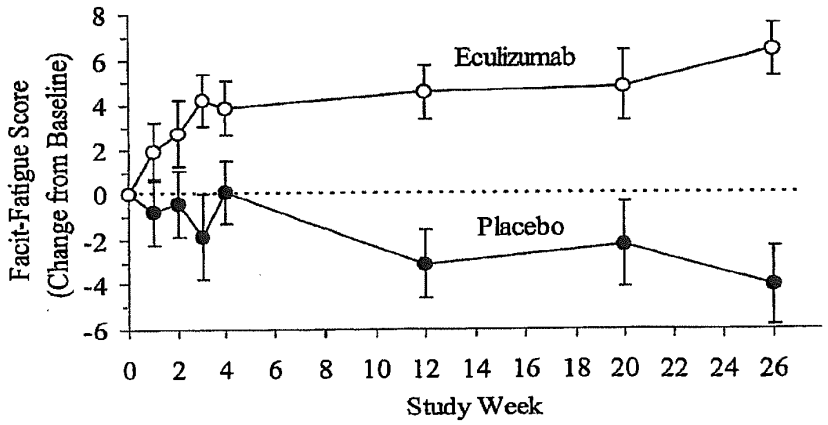

Facit-Fatigue Score
(Change from Baseline)
8
6
4
2
0
-2
-4
-6
Eculizumab
Placebo
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Study Week

FIGURE 3

Form 1002 - 1: Public inventor(s)

Designation of inventor 123

User reference: P2913 EP/3
Application No:

Public

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Date:	**24 October 2016**
Signed by:	**/Dr. Joachim Wachenfeld/**
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Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1028
Page 054

New EP-Divisional Patent Application
based on EP 11 00 1632.6
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A.[1,2] Mutations in PIG-A lead to an early block in the synthesis of glycosylphosphatidylinositol (GPI)-anchors, which are required to tether many proteins to the cell surface. Consequently, PNH blood cells have a partial (type II) or complete (type III) deficiency of GPI-anchored proteins.

Intravascular hemolysis is a prominent feature of PNH and a direct result of the absence of the GPI-anchored complement regulatory protein CD59.[3,4] Under normal circumstances, CD59 blocks the formation of the terminal complement complex (also called the membrane attack complex) on the cell surface, thereby preventing erythrocyte lysis and platelet activation.[5-8] Excessive or persistent intravascular hemolysis in PNH patients not only results in anemia (normal ranges of hemoglobin are 14-18 g/dL for men and 12-16 g/dL for women, and persons with lower levels are considered to be anemic), but also hemoglobinuria and clinical sequelae related to the release of the erythrocyte contents into the circulation: fatigue, thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9,10,21,22] Indeed, impaired quality of life in PNH is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate erythrocyte hemoglobin levels. There have been no therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH.

Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5.[11] In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards.

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SUMMARY

The present pivotal, phase III study, Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH), evaluated the effect of eculizumab on the stabilization of hemoglobin levels and transfusion requirements during 6 months of treatment in a cohort of 87 transfusion-dependent PNH patients. Measures of intravascular hemolysis and quality of life were also assessed. This is the first placebo controlled study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effects due to anemia.

It has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab. Furthermore, these improvements in the quality of life were independent of transfusion. The improved aspects include, e.g., global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss and insomnia. Improvement was also seen in nausea and vomiting, diarrhea, constipation, and financial difficulties but did not reach the level of statistical significance. Because the treated patients remained anemic throughout their treatment, it was unexpected that all of these improvements would have been seen because they were previously thought to be a result of the patient being anemic. Although not wishing to be bound by any theory, it appears that some of the symptoms are likely due, at least in part, to hemolysis and release of hemoglobin into the bloodstream and do not result solely from the patient being anemic. The treatment with eculizumab decreases the amount of lysis thereby limiting hemoglobin release into the bloodstream, thereby apparently resulting in the improvements seen in the treated patients' quality of life. The results presented herein indicate that any treatment that decreases hemolysis in a patient will result in an improvement in the quality of life of said patient.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits complement or inhibits formation of C5b-9.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits

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intravascular hemolysis. In certain embodiments, said method results in a greater than 30% reduction in LDH in said patient.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis, wherein said patient remains anemic. In certain embodiments, said method results in a greater than 30% reduction in LDH in said patient.

In certain aspects, the application provides a method of prolonging the health-adjusted life expectancy of a patient comprising administering to said patient in need thereof a compound which inhibits formation of C5b-9. In certain embodiments, said patient is anemic. In certain embodiments, said patient remains anemic following treatment. In certain embodiments, said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman. In certain embodiments, said patient suffers from paroxysmal nocturnal hemoglobinuria.

In certain aspects, the application provides a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is pexelizumab. In certain embodiments, the pharmaceutical formulations of the application may be administered to a subject, particularly a subject having PNH.

In certain aspects, the application provides a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria by administering a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is pexelizumab. In certain embodiments, the pharmaceutical formulations of the application may be administered to a subject, particularly a subject having PNH.

In certain aspects, the application provides kits comprising a pharmaceutical composition of the application. In some embodiments, the kit further comprises at least one component of a closed sterile system. Components of the closed sterile system

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include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. In a related embodiment, the kit comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

In certain embodiments, said quality of life is measured by a FACIT-Fatigue score. In certain embodiments, the FACIT-Fatigue score increases by at least 3 points. In certain embodiments, the FACIT-Fatigue score increases by ≥ 4 points.

In certain embodiments, said quality of life is measured by an EORTC QLQ-C30 score. In certain embodiments, said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score. In certain embodiments, said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia. In certain embodiments, said aspect of quality of life is fatigue.

In certain embodiments, said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. In certain embodiments, said compound is a steroid that suppresses complement.

In certain embodiments, said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors. In certain embodiments, said compound is an antibody or an active antibody fragment. In certain embodiments, said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

In certain embodiments, said antibody or an active antibody fragment thereof binds C5. In certain embodiments, said antibody or active antibody fragment blocks C5 cleavage. In certain embodiments, said antibody or active antibody fragment inhibits the formation of C5b-9. In certain embodiments, said antibody is eculizumab. In certain embodiments, said antibody or active antibody fragment is administered for at least 6

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months. In certain embodiments, said patient has aplastic anemia or myelodysplastic syndrome.

In certain embodiments, said antibody that binds C5 or an active antibody fragment thereof is administered in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the single unit dosage form is lyophilized. In certain embodiments, the single unit dosage form is a sterile solution. In certain embodiments, the single unit dosage form is a preservative free formulation. In certain embodiments, the 300 mg single-use dosage form comprises 30 ml of a 10 mg/ml sterile, preservative free solution.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises an altered constant region, wherein said antibody or antigen-binding fragment exhibits decreased effector function relative to an anti-CDCP1 antibody with a native constant region. In certain embodiments, decreased effector function comprises one or more properties of the following group: a) decreased antibody-dependent cell-mediated cytotoxicity (ADCC), and b) decreased complement dependent cytotoxicity (CDC), compared to an anti-CDCP1 antibody with a native constant region. In certain embodiments, the altered constant region comprises a G2/G4 construct in place of the G1 domain.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3. In certain embodiments, the pharmaceutical composition comprises eculizumab. In certain embodiments, the pharmaceutical composition comprises pexelizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ ID NO: 4.

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In certain embodiments, said patient is anemic. In certain embodiments, said patient remains anemic following treatment. In certain embodiments, said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

In certain embodiments, said health-adjusted life expectancy is measured according to a unit selected from the group consisting of Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, or Years of healthy life.

In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one day. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least week. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one month. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one year.

In certain embodiments, the pharmaceutical composition is in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the pharmaceutical composition is lyophilized. In certain embodiments, the pharmaceutical composition is a sterile solution. In certain embodiments, the pharmaceutical composition is a preservative free formulation. In certain embodiments, the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. In certain embodiments, the pharmaceutical composition comprises an antibody that binds C5 or an active antibody fragment thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B show that eculizumab treatment decreases intravascular hemolysis and increases PNH type III erythrocytes. Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels. Figure 1B shows the mean proportion of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients.

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Figure 2 shows the effect of eculizumab treatment on transfusion requirements in PNH patients. This is a Kaplan-Meier plot of time to first transfusion for eculizumab- and placebo-treated patients from baseline through week 26. The P value is from the log rank analysis.

Figure 3 shows the effect of eculizumab on fatigue assessed by the FACIT-Fatigue Instrument. Quality of Life scores were assessed using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. Values for change from baseline to 26 weeks represent least-square means. A positive change indicates an improvement and a negative change indicates deterioration in the FACIT-Fatigue measures of quality of life.

DETAILED DESCRIPTION

I. Definitions

The term "derived from" means "obtained from" or "produced by" or "descending from".

The term "genetically altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a native antibody. Because of the relevance of recombinant DNA techniques to this application, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and range from the changing of just one or a few amino acids to the complete redesign of, for example, the variable or constant region. Changes in the constant region will, in general, be made in order to improve or alter characteristics, such as complement fixation, interaction with membranes and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics.

The term "an antigen-binding fragment of an antibody" refers to any portion of an antibody that retains the binding utility to the antigen. An exemplary antigen-binding fragment of an antibody is the heavy chain and/or light chain CDR, or the heavy and/or light chain variable region.

The term "homologous," in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that have at least about 85%, at least 90%, at least 95%, or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison

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method and/or by visual inspection. In certain embodiments, the "homolog" exists over a region of the sequences that is about 50 residues in length, at least about 100 residues, at least about 150 residues, or over the full length of the two sequences to be compared.

Methods of determining percent identity are known in the art. "Percent (%) sequence identity" with respect to a specified subject sequence, or a specified portion thereof, may be defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul et al., J. Mol. Biol. 215:403-410 (1997); http://blast.wustl.edu/blast/README.htm- l) with search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A "% identity value" is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported.

II. Overview

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH"), more specifically to improving certain aspects of quality of life which are impaired in PNH patients, and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased

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enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

III The Complement System

The complement system, useful complement inhibitors, and use of these inhibitors to treat PNH and other patients are more fully described in PCT Patent Application PCT/US2005/003225 filed February 3, 2005 and published as International Publication Number WO 2005/074607 A2 on August 18, 2005, the contents of which are incorporated herein by reference in their entirety.

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most

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important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., 1991). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658), with four amino acids deleted between the two.

C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, 1977; Wetsel and Kolb, 1982) and acid treatment

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(Yamamoto and Gewurz, 1978; Vogt et al., 1989) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction.

IV Measures of Quality of Life

Various measurements exist to assess quality of life and the effect of medical interventions on quality of life for example the Mini-Mental State Examination (MMSE), the Short Test of Mental Status, the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire, the FACIT questionnaires and subscales including fatigue and anemia, the Likert Scale, and Borg Scale (Tombaugh, et al., J. Am. Geriatr. Soc. 40:922, 1992; Cummings, JAMA. 269(18):2420, 1993; Crum, et al., JAMA. 269(18):2386, 1993; Folstein, et al., J. Psychiat. Res. 12:189,1975; Kokmen, et al., Mayo Clin. Proc. 62:281, 1987; Tang-Wai, et al., Arch. Neurol. 60:1777, 2003; Tamburini, Ann.

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Oncol. 12(Suppl. 3):S7, 2001; Webster et al., Health and Quality of Life Outcomes. 1:79, 2003, www.hqlo.com/content/I/I/79; Grant, et al., Chest. 116:1208, 1999; and www.qolid.org). Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In certain embodiments, improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 is measured by the Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System. In certain embodiments, improvement in quality of life is measured by: a) full scales; b) stand-alone subscales; and c) symptom indices.

In certain embodiments, improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 is measured by a European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire. In certain embodiments, the EORTC questionnaire is the QLQ-C30.

In certain embodiments, improvement in quality of life is measured by the health-adjusted life expectancy (HALE) index as described in Wilkins, R. and Adams, OB., Am J Public Health, 73:1073-1080 (1983). Health-adjusted life expectancy is an average of the quality-adjusted life years (QALY) for a given population and can be used to evaluate the therapeutic value of a medical intervention. Quality-adjusted life years is a health index that weighs each year of life on a scale from 1 to 0 (Weinstein MC and Stason WB, N Engl J Med, 296:716-721 (1977)). Perfect health is rated as 1, death is rated as 0, and disability and pain are rated based on severity. QALY is determined by multiplying the number of years at each health status.

In certain embodiments, improvement in quality of life is measured by the following instruments: Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, and Years of healthy life. These measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In one embodiment, the disclosed methods improve the quality of life of a patient for at least one day, at least one week, at least two weeks, at least three weeks, at least one

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month, at least two months, at least three months, at least 6 months, at least one year, at least 18 months, at least two years, at least 30 months, or at least three years, or the duration of treatment.

In certain embodiments, the symptoms used to measure quality of life are scaled for intensity. In certain embodiments, the symptoms are scaled for frequency. In certain embodiments, the symptoms are scaled for intensity and frequency.

In certain aspects, the application provides a method for prolonging the health-adjusted life expectancy of a subject comprising administering to the subject a compound which inhibits complement or inhibits formation of C5b-9. The above measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality-adjusted life expectancy due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In one embodiment, the disclosed methods prolong the health-adjusted life expectancy in a subject by at least one day, at least one week, at least two weeks, at least three weeks, at least one month, at least two months, at least three months, at least 6 months, at least one year, at least 18 months, at least two years, at least 30 months, or at least three years as measured by the health-adjusted life expectancy (HALE) index as described in Wilkins et al. Am J Public Health, 73:1073-1080 (1983). Health-adjusted life expectancy is an average of the quality-adjusted life years (QALY) for a given population and can be used to evaluate the therapeutic value of a medical intervention. Quality-adjusted life years is a health index that weighs each year of life on a scale from 1 to 0 (Weinstein et al., N Engl J Med, 296:716-721 (1977)). Perfect health is rated as 1, death is rated as 0, and disability and pain are rated based on severity. QALY is determined by multiplying the number of years at each health status.

V Inhibitors of the Complement Cascade

In certain embodiments, any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the present methods. In certain embodiments, a complement inhibitor may be a small molecule (up to 6,000 Da in molecular weight), a nucleic acid or nucleic acid analog, a peptidomimetic, or a macromolecule that is not a nucleic acid, a serine protease inhibitor, or a protein. These agents include, but are not limited to, small organic molecules, RNA

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aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, Mass.), L-RNA aptamers, Spiegelmers, antisense compounds, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors.

In certain embodiments, a complement inhibitor may be a protein or protein fragment. Proteins are known which inhibit the complement cascade, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated neural injury, and C3a and C5a production. In certain embodiments, a complement inhibitor may be naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

In certain embodiments, a complement inhibitor may be an antibody capable of inhibiting complement, such as an antibody that can block the formation of MAC. For example, an antibody complement inhibitor may include an antibody that binds C5. Such anti-C5 antibodies may directly interact with C5 and/or C5b, so as to inhibit the formation of and/or physiologic function of C5b.

Suitable anti-C5 antibodies are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and inhibits cleavage into C5a and C5b thereby decreasing the formation not only of C5a but also the downstream complement components.

The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, J Clin Invest. 1971 Mar;50(3):606-16; Wurzner, et al., Complement Inflamm. 8:328-340, 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays

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known in the art can also be used. Using assays of these or other suitable types, candidate antibodies capable of inhibiting complement such as anti-C5 antibodies, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the application and 2) determine the appropriate dosage levels of such compounds.

An antibody capable of inhibiting complement such as an antibody that binds C5 affecting C5b is preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25% as compared to that in the absence of the antibody that binds C5) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. Such concentrations can be conveniently determined by measuring the cell-lysing ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid. Accordingly, a specific concentration for an antibody that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer (eds), "Experimental Immunochemistry, 2d Edition", 135-240, Springfield, IL, CC Thomas (1961), pages 135-139, or a conventional variation of that assay such as the chicken erythrocyte hemolysis method described below.

Specific antibodies capable of inhibiting complement, such as an antibody that binds C5, are relatively specific and do not block the functions of early complement components. In particular, such specific agents will not substantially impair the opsonization functions associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 convertases and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, specific antibodies capable of inhibiting complement such as an antibody that binds C5 do not substantially interfere with the cleavage of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios

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by the actions of the C3 convertases. Such measurements are informative because C3a and C3b levels will be reduced (compared to a matched sample without the antibody capable of inhibiting complement such as an antibody that binds C5) if cleavage is interfered with by an antibody capable of inhibiting complement such as an antibody that binds C5.

In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g., that sold by Quidel Corporation, San Diego, Calif., according to the manufacturer's specifications. Particularly specific antibodies capable of inhibiting complement such as an antibody that binds C5 produce essentially no reduction in body fluid C3a levels following complement activation when tested in such assays.

Certain antibodies of the disclosure will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. As discussed above, in a particular embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the action of C3b.

A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and inhibits cleavage. This decreases the formation of both C5a and C5b while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies which are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include a preferred whole antibody (now named eculizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., Mol. Cell. Probes. 1:141-149 (1987)). Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, single-chain antibody, and Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., Nature 321:522-5 (1986)), chimerized, or deimmunized. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies.

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In specific embodiments, a therapeutic agent of the disclosure comprises an antibody or antibody fragment. Antibodies and fragments thereof may be made by any conventional method, such as those methods described herein. Antibodies are found in multiple forms, e.g., IgA, IgG, IgM, etc. Additionally, antibodies can be engineered in numerous ways. They can be made as single-chain antibodies (including small modular immunopharmaceuticals or SMIPs™), Fab and $F(ab')_2$ fragments, etc. Antibodies can be humanized, chimerized, deimmunized, or fully human. Numerous publications set forth the many types of antibodies and the methods of engineering such antibodies. For example, see U.S. Patent Nos. 6,355,245; 6,180,370; 5,693,762; 6,407,213; 6,548,640; 5,565,332; 5,225,539; 6,103,889; and 5,260,203.

This invention provides fragments of anti-C5 antibodies, which may comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8:1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment of an antibody yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" refers to the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although likely at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a

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free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds. (Springer-Verlag: New York, 1994), pp. 269-315.

SMIPs are a class of single-chain peptide engineered to include a target binding region, effector domain (CH2 and CH3 domains). *See, e.g.*, U.S. Patent Application Publication No. 20050238646. The target binding region may be derived from the variable region or CDRs of an antibody, e.g., an antibody that binds C5 of the application. Alternatively, the target binding region is derived from a protein that binds C5.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993).

It is well known that the binding to a molecule (or a pathogen) of antibodies with an Fc region assists in the processing and clearance of the molecule (or pathogen). The Fc portions of antibodies are recognized by specialized receptors expressed by immune effector cells. The Fc portions of IgG1 and IgG3 antibodies are recognized by Fc receptors present on the surface of phagocytic cells such as macrophages and neutrophils, which can thereby bind and engulf the molecules or pathogens coated with antibodies of these isotypes (C. A. Janeway et al., Immunobiology 5th edition, page 147, Garland Publishing (New York, 2001)).

This disclosure also provides monoclonal anti-C5 antibodies. A monoclonal antibody can be obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal

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antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., Nature 256:495-497 (1975), or may be made by recombinant DNA methods (*see, e.g.*, U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature 352:624-628 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example.

A description of the preparation of a mouse anti-human-C5 monoclonal antibody with specific binding characteristics is presented in U.S. Patent Application Publication No. 20050226870. Wurzner et al., Complement Inflamm. 8:328-340 (1991), describe the preparation of other mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

Other antibodies specifically contemplated are "oligoclonal" antibodies. As used herein, the term "oligoclonal" antibodies" refers to a predetermined mixture of distinct monoclonal antibodies. *See, e.g.*, PCT publication WO 95/20401; U.S. Patent Nos. 5,789,208 and 6,335,163. In one embodiment, oligoclonal antibodies consisting of a predetermined mixture of antibodies against one or more epitopes are generated in a single cell. In other embodiments, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (*e.g.*, PCT publication WO 04/009618). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (*e.g.*, C5). In view of the assays and epitopes disclosed herein, those skilled in the art can generate or select antibodies or mixtures of antibodies that are applicable for an intended purpose and desired need.

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In certain embodiments that include a humanized and/or chimeric antibody, one or more of the CDRs are derived from an anti-human C5 antibody. In a specific embodiment, all of the CDRs are derived from an anti-human C5 antibody. In another specific embodiment, the CDRs from more than one anti-human C5 antibody are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first anti-human C5 antibody combined with CDR2 and CDR3 from the light chain of a second anti-human C5 antibody, and the CDRs from the heavy chain may be derived from a third anti-human C5 antibody. Further, the framework regions may be derived from one of the same anti-human C5 antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody. Human or humanized antibodies are specific for administration to human patients.

In certain embodiments, single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single chain antibodies, comprising portions derived from different species, are also encompassed by the present disclosure as antigen-binding fragments of an antibody. The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., U.S. Pat. Nos. 4,816,567 and 6,331,415; U.S. Pat. No. 4,816,397; European Patent No. 0,120,694; WO 86/01533; European Patent No. 0,194,276 B1; U.S. Pat. No. 5,225,539; and European Patent No. 0,239,400 B1. See also, Newman et al., BioTechnology 10:1455-1460 (1992), regarding primatized antibody. See, e.g., Ladner et al., U.S. Pat. No. 4,946,778; and Bird et al., Science 242:423-426 (1988), regarding single chain antibodies.

In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen-binding function of a corresponding full-length antibody (such as for example, ability of antibody that binds C5 to bind C5).

General methods for the immunization of animals (in this case with C5 and/or C5b, etc.), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of

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hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case the immunogen or a molecule containing the immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz et al., Cellular Immunol. 127:337-351 (1990); Wurzner et al., Complement Inflamm. 8:328-340 (1991); and Mollnes et al., Scand. J. Immunol. 28:307-312 (1988).

VI Methods of Treatment

Methods of the application may be used to treat paroxysmal nocturnal hemoglobinuria associated symptoms. Methods of the application may be used to treat anemia associated symptoms. Treatment of paroxysmal nocturnal hemoglobinuria and/or anemia may be administered by standard means. Treatments of the application may be used in combination with other treatments of the application or known treatments for paroxysmal nocturnal hemoglobinuria and/or anemia. Treatments of the application may be co-administered with other treatments that treat symptoms of paroxysmal nocturnal hemoglobinuria and/or anemia.

VII Pharmaceutical Formulations and Uses

Methods of administration of small molecules, proteins, and nucleic acids are well-known to those of skill in the art. Methods of administration of antibodies are well-known to those of skill in the art. To achieve the desired inhibition, the antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood. The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing

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physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/mL to about 500 μg/mL. However, greater amounts may be required for extreme cases and smaller amounts may be sufficient for milder cases.

In certain embodiments, the pharmaceutical composition is in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the pharmaceutical composition is lyophilized. In certain embodiments, the pharmaceutical composition is a sterile solution. In certain embodiments, the pharmaceutical composition is a preservative free formulation. In certain embodiments, the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. In certain embodiments, the antibody is administered according to the following protocol: 600 mg via 25 to 45 minute IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter. Antibody is administered via IV infusion over 25 to 45 minute.

Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired but an intravenous route will be the most preferable. Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like. In certain embodiments, complement inhibitors such as eculizumab may be administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Administration of the antibodies capable of inhibiting complement such as an antibody that binds C5 will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if

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desired and practicable for the particular antibody capable of inhibiting complement to be administered. Antibodies capable of inhibiting complement such as an antibody that binds C5 can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular antibody capable of inhibiting complement being administered. Doses of antibodies capable of inhibiting complement such as an antibody that binds C5 will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

In certain embodiments, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints with the dosage levels adjusted as needed to achieve the desired clinical outcome. In certain embodiments, treatment is administered in multiple dosages over at least a week. In certain embodiments, treatment is administered in multiple dosages over at least a month. In certain embodiments, treatment is administered in multiple dosages over at least a year. In certain embodiments, treatment is administered in multiple dosages over the remainder of the patient's life.

The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the therapeutic of the disclosure, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the therapeutic of the disclosure in the body fluid may be monitored during the course of treatment.

In certain embodiments, the frequency of administration may be adjusted according to an assay measuring cell-lysing ability of complement present in one or more of the patient's body fluids. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the application means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

For the treatment of hemolytic diseases such as PNH by systemic administration of an antibody capable of inhibiting complement such as an antibody that binds C5 (as opposed to local administration), administration of a large initial dose is specific, i.e., a

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single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

Formulations particularly useful for antibody-based therapeutic agents are also described in U.S. Patent App. Publication Nos. 20030202972, 20040091490 and 20050158316. In certain embodiments, the liquid formulations of the application are substantially free of surfactant and/or inorganic salts. In another specific embodiment, the liquid formulations have a pH ranging from about 5.0 to about 7.0. In yet another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from about 1 mM to about 100 mM. In still another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from 1 mM to 100 mM. It is also contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g., arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzing liquid formulations can be found, for example, in PCT publications WO 03/106644, WO 04/066957, and WO 04/091658.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions of the application.

In certain embodiments, formulations of the subject antibodies are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside microorganisms and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a

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single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin.

Formulations of the subject antibodies include those suitable for oral, dietary, topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), ophthalmologic (e.g., topical or intraocular), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), rectal, and/or intravaginal administration. Other suitable methods of administration can also include rechargeable or biodegradable devices and controlled release polymeric devices. Stents, in particular, may be coated with a controlled release polymer mixed with an agent of the application. The pharmaceutical compositions of this disclosure can also be administered as part of a combinatorial therapy with other agents (either in the same formulation or in a separate formulation).

The amount of the formulation which will be therapeutically effective can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. The dosage of the compositions to be administered can be determined by the skilled artisan without undue experimentation in conjunction with standard dose-response studies. Relevant circumstances to be considered in making those determinations include the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms. For example, the actual patient body weight may be used to calculate the dose of the formulations in milliliters (mL) to be administered. There may be no downward adjustment to "ideal" weight. In such a situation, an appropriate dose may be calculated by the following formula: Dose (mL) = [patient weight (kg) x dose level (mg/kg)/ drug concentration (mg/mL)]

To achieve the desired treatment results, anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require

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different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

Other therapeutics of the disclosure can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and *in vivo* half-life of the particular therapeutic being administered.

Doses of therapeutics of the disclosure will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

The formulations of the application can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the antibody capable of inhibiting complement and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material may include a label which indicates that the formulation is for use in the treatment of hemolytic diseases such as PNH. Although antibodies are preferred, especially anti-C5 antibodies which have already been shown to be safe and effective at decreasing the accumulation of downstream complement components in persons, the use of other complement inhibitors is also contemplated by this disclosure. The pharmaceutical formulations and uses of the disclosure may be combined with any known complement inhibitors or hemolytic diseases treatments known in the art.

In certain aspects, the application provides kits comprising a pharmaceutical composition of the application. In some embodiments, the kit further comprises at least one component of a closed sterile system. Components of the closed sterile system include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. In a related embodiment, the kit comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

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EXEMPLIFICATION

METHODS

Patient Selection

The TRIUMPH trial consisted of a 2-week screening period, an observation period of up to 3 months duration, and a 26-week treatment period.

During the screening period, patients were evaluated with respect to inclusion and exclusion criteria. Men and women, 18 years or older, diagnosed as having PNH with a type III erythrocyte population of ≥10%, and who had received at least 4 transfusions in the previous 12 months were eligible. Concomitant administration of erythropoietin, immunosuppressants, corticosteroids, coumadin, low molecular weight heparin, iron supplements, and folic acid were not reasons for exclusion, provided the doses were steady prior to the first visit and throughout the duration of the study. Because of the increased frequency of neisserial infections in individuals genetically deficient in terminal complement proteins, all patients were vaccinated against *Neisseria meningitides*. Patients were to avoid conception. The protocol was approved by an Investigational Review Board at each clinical site and written informed consent was obtained from all patients enrolled.

Patients transfused with a mean pre-transfusion hemoglobin level >10.5 g/dL over the previous 12 months, and those who showed evidence of having a suppressed immune response, complement deficiency, or active bacterial infection, including any history of meningococcal disease, were excluded from the study. Patients were also not eligible if they had previously received a bone marrow transplant or if they had participated in another trial or received another investigational drug within 30 days of the first visit. An individualized transfusion algorithm was calculated for each patient based on their prior 12-month transfusion history; the written algorithm documented the number of packed red blood cell (PRBC) units transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during observation and treatment periods.

Each patient considered eligible entered an observation period of up to 13 weeks in order to confirm their PBRC transfusion dependence. At least one transfusion --termed the "qualifying" transfusion-- during the 13-week observation period at a hemoglobin value at or below 9 g/dL with symptoms, or at or below 7 g/dL with or without symptoms, in accordance with the transfusion algorithm indicated for each patient, was a requirement to

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proceed to randomization. The hemoglobin value at which each individual's qualifying transfusion was administered, was defined as the hemoglobin "set point" for that individual for the purpose of the primary efficacy variable. A platelet count ≥100,000/mL and a LDH level ≥1.5 times the upper limit of the normal range were also required either at screening or during the observation period for eligibility.

Study Design

Patients were randomly assigned on a one-on-one basis to receive either placebo or eculizumab (Soliris™, Alexion Pharmaceuticals, Inc.) within 10 days of the qualifying transfusion. Study medication was dosed in a blinded fashion as follows: 600 mg eculizumab for patients randomly assigned to active drug, or placebo for those patients randomly assigned to placebo, respectively via IV infusion every 7±1 days for 4 doses; followed by 900 mg eculizumab, or placebo, respectively, via IV infusion 7±1 day later; followed by a maintenance dose of 900 mg eculizumab, or placebo, respectively, via IV infusion every 14±2 days for a total of 26 weeks of treatment.

Measures of Clinical Efficacy

There were two co-primary endpoints in the study: (1) stabilization of hemoglobin levels, defined as a hemoglobin value maintained above the individual hemoglobin set point in the absence of transfusions for the entire 26-week treatment period, and (2) reduction in units of PRBCs transfused during the 26-week treatment phase of the study. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia and reached their individualized, pre-determined "set point". Pre-specified secondary endpoints included transfusion avoidance, hemolysis as measured by LDH area under the curve from baseline to 26 weeks, and QoL changes as measured from baseline to 26 weeks using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument.[13] Pre-specified exploratory analyses included assessment of the EORTC QLQ-C30 instrument,[14] the change in LDH from baseline through week 26, and thrombosis. Other pre-specified measurements included pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. Time to first transfusion during the 26-week treatment phase and the proportion of PNH type III blood cells were also assessed.

Safety Assessments

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Treatment-emergent adverse events, clinical laboratory tests (e.g., serum chemical analyses and complete blood counts), electrocardiogram data, and vital signs were assessed. Adverse events were defined using the MedDRA preferred terms and tabulated as incidence rates per treatment group.

Statistical Analysis

For co-primary endpoints, analyses were performed according to the intention to treat using the data from all patients who were randomized and received study drug; stabilization of hemoglobin levels was analyzed using the Fisher's exact test and total PRBC units transfused were analyzed with the Wilcoxon's rank sum test. For comparison of treatment effect on transfusion avoidance, the Fisher's exact test was used on the incidence and the log rank test was used for time to first transfusion. For LDH area under the curve the Wilcoxon's rank sum test was used.

Quality of life measure of fatigue was assessed using the scoring guidelines for the FACIT-Fatigue instrument.[15] Assessment of quality of life measures based on the EORTC QLQ-C30 instrument was conducted in accordance with the appropriate scoring guidelines.[16] The changes of FACIT-Fatigue and EORTC QLQ-C30 scores from baseline through 26 weeks were analyzed using a mixed model, with baseline as covariate, treatment and time as fixed effects, and patient as a random effect. Changes in LDH levels and PNH type III erythrocytes from baseline through 26 weeks were analyzed using the same mixed model. Two-sided tests were used for all analyses. The adverse events and long-term safety checklist were tabulated separately and compared between treatments using the Fisher's exact test. A p-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Characteristics of Patients

A total of 115 PNH patients were screened. Six patients did not meet the inclusion/exclusion criteria during the screening period. Twenty-one other patients did not receive a qualifying transfusion and were not randomized into the treatment phase. One patient who did not meet the inclusion criteria was inadvertently randomized, but did not receive study medication. Thus 87 hemolytic PNH patients (35 men and 52 women) were enrolled and randomized to receive either eculizumab (N=43) or placebo (N=44), exceeding the original target of 75 randomized patients.

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Patient characteristics were similar in the eculizumab- and placebo-treated cohorts: median age, 41 (range 20-85) and 35 (range 18-78) years; median duration of PNH, 4.2 (range 0.8 to 29.7) and 9.2 (range 0.4 to 38.3) years; patients with history of aplastic anemia, 4 and 11; history of myelodysplastic syndrome, 1 and 0; and history of thrombosis, 9 (16 events) and 8 (11 events). Stable usage of concomitant medications at baseline in the eculizumab- and placebo-treated groups included the following: erythropoietin, 3 patients and 0 patients; cyclosporine, 1 and 1; anticoagulants (coumarins or heparins) 21 and 11; and steroids (glucocorticoids or androgenic steroids), 12 and 12, respectively.

Of the 87 patients randomized, 85 completed the trial. Two patients who did not complete the trial had been randomized to the eculizumab arm: one patient discontinued due to the inconvenience of travel to the study site and the second patient became pregnant. Ten patients in the placebo-treatment group discontinued infusions, in all cases due to perceived lack of efficacy, but they remained in the study for monitoring purposes.

Pharmacokinetics/Pharmacodynamics

In 42 of the 43 eculizumab-treated patients the levels of drug during the maintenance period (900 mg every 2 weeks±2 days) were sufficient to completely block serum hemolytic activity (mean trough value at week 26 of 101.8 μg/mL). A single patient did not sustain therapeutic trough levels of eculizumab and demonstrated a breakthrough in complement blockade during the last few days of each dosing interval. These breakthroughs were clinically manageable and quickly resolved following the next dose.

Hemolytic Efficacy Variables

The impact of terminal complement inhibition with eculizumab on chronic intravascular hemolysis in PNH patients was demonstrated in this study by an immediate (one week) and sustained decrease in mean levels of LDH (Figure 1A). The median LDH area under the curve during the 26-week study period was reduced 85.8% in eculizumab- relative to placebo-treated patients ($P<0.001$). The mean LDH level decreased from 2199.7±157.7 IU/L at baseline to 327.3±67.6 IU/L by 26 weeks in eculizumab-treated patients while levels in placebo-treated patients remained consistently elevated with values of 2259.0±158.5 IU/L at baseline and 2418.9±140.3 IU/L at 26 weeks ($P<0.001$, for eculizumab versus placebo). A second biochemical measure of hemolysis, serum aspartate aminotransferase (AST), also showed a statistically significant improvement following eculizumab- versus placebo-treatment (data not shown). Haptoglobin levels were statistically significantly increased in eculizumab- as compared to placebo-treated patients

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but mean levels of haptoglobin were still below normal levels in eculizumab-treated patients (data not shown).

Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels (±standard error) from baseline (study week 0) to week 26 for both eculizumab- and placebo-treated patient populations. Screening occurred up to 3 months prior to study week 0. The upper limit of the normal range (103-223 IU/L) for LDH is indicated by a dashed line. LDH was reduced to a mean level just above the upper limit of normal at week 26 for eculizumab-treated patients; 15 of 41 patients who completed the study demonstrated LDH levels within the normal range. All placebo-treated patients remained at least 5 times above the upper limit of normal at week 26. The P value is based on a mixed model analysis from baseline through week 26. Figure 1B shows the mean proportion (±standard error) of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients. The screening visit occurred up to 3 months prior to study week 0. The P value is based on a mixed model analysis from baseline through week 26.

A corollary to the reduction in intravascular hemolysis during eculizumab treatment was an observed increase in the PNH type III erythrocyte population (Figure 1B). The mean proportions of type III erythrocytes increased from 28.1±2.0% at baseline to 56.9±3.6% by week 26 for eculizumab-treated patients while proportions in the placebo group remained constant with mean values of 35.7±2.8% before treatment to 35.5±2.8% at 26 weeks ($P<0.001$, for eculizumab versus placebo). By contrast, the proportions of PNH type III granulocytes and monocytes did not change significantly between the treatment groups during the treatment period and were greater than 90% at week 26.

CLINICAL EFFICACY

Co-primary endpoints

The co-primary efficacy endpoints in the TRIUMPH trial were stabilization of hemoglobin levels and reduction in PRBC units transfused. At the end of the treatment period, 48.8% of eculizumab-treated patients had maintained levels of hemoglobin above the pre-specified set-point (median set-point value of 7.7 g/dL for both treatment groups) in the absence of transfusions, whereas stabilization of hemoglobin did not occur in any of the patients in the placebo group ($P<0.001$; Table 1). By week 26, the median of PRBC units transfused per patient was 0 in the eculizumab group and 10.0 in the placebo cohort

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(P<0.001), while the mean of PRBC units transfused was 3.0 and 11.0 in the eculizumab and placebo cohorts, respectively. In the 6-month period prior to the study, the median of PRBC units transfused per patient was 9.0 in the eculizumab cohort and 8.5 in placebo patients while the mean of PRBC units transfused was 9.6±0.6 and 9.7±0.7, respectively. Mean hemoglobin levels were similar between the treatment groups at baseline (10.0±1.8 g/dL in eculizumab-treated patients and 9.7±1.8 g/dL in placebo-treated patients) and did not substantially change by week 26 (10.1±2.5 g/dL and 8.9±2.2 g/dL in eculizumab and placebo cohorts, respectively).

The median time to first transfusion was not reached during the study period in eculizumab-treated patients (it was greater than 26 weeks) while the placebo group reached the median time to first transfusion in only 4 weeks (P<0.001; Figure 2). Transfusion avoidance was achieved in 51.2% and 0% of the eculizumab and placebo cohorts, respectively (P<0.001). By the end of the 26-week treatment period, the total PRBC units transfused were 131 in eculizumab-treated patients versus 482 in the placebo group (Table 1). By contrast, in the 6-month period prior to the study, total PRBC units transfused in the eculizumab- and placebo-cohorts were 413 and 417, respectively.

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

	PRE-TREATMENT HISTORY*		TREATMENT PERIOD		
	Placebo	Eculizumab	Placebo	Eculizumab	P Value
†Stabilization of Hemoglobin Levels in the absence of transfusions (percent of patients)	NA	NA	0	48.8	<0.001‡
†Units Transfused per Patient					
Median	8.5	9.0	10	0	<0.001§
Mean±SE	9.7±0.7	9.6±0.6	11.0±0.83	3.0±0.67	
Total Units Transfused per Group	417	413	482	131	

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

Assessments of quality of life in PNH patients during eculizumab treatment were performed using two different instruments, FACIT-Fatigue and the EORTC QLQ-C30. Eculizumab-treated patients showed a mean increase (improvement) in the FACIT-Fatigue score of 6.4±1.2 points from baseline to 26-weeks while the mean score in placebo patients decreased by 4.0±1.7 points, a total difference between the treatment groups of 10.4 points (Figure 3). Mixed model analysis of covariance demonstrated a statistically significant difference between treatment groups (P<0.001).

For the EORTC instrument, improvements were observed with eculizumab-treatment in each subscale. Statistically significant improvements with eculizumab-compared with placebo-treated groups were observed in the following quality of life

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subscales (Table 2): global health status (P<0.001), physical functioning (P<0.001), emotional functioning (P=0.008), cognitive functioning (P=0.002), role functioning (P<0.001), social functioning (P=0.003), fatigue (P<0.001), pain (P=0.002), dyspnea (P<0.001), appetite loss (P<0.001), and insomnia (P=0.014). The improvements with eculizumab treatment in the other scales, including nausea and vomiting, diarrhea, constipation, and financial difficulties, did not reach statistical significance.

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

	Mean Change from Baseline to Week 26*			
	Placebo	**Eculizumab**	**Absolute Difference**	**P Value†**
Global Health Status	-8.5	10.9	19.4	<0.001
Functional				
Role	-6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.6	=0.003
Cognitive	-6.1	7.9	14.0	=0.002
Physical	-3.5	9.4	13.0	<0.001
Emotional	-3.7	7.5	11.2	=0.008
Symptoms/Single Items				
Fatigue	10.0	-16.9	27.0	<0.001
Pain	5.3	-12.3	17.6	=0.002
Dyspnea	8.9	-7.9	16.9	<0.001
Appetite Loss	3.3	-10.3	13.6	<0.001
Insomnia	4.9	-7.9	12.8	=0.014

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Financial Difficulties	0.0	-10.3	10.3	=0.186
Constipation	0.0	-6.3	6.3	=0.199
Nausea/vomiting	2.8	-0.4	3.2	=0.056
Diarrhea	5.7	4.8	0.9	=0.147

*A positive change indicates an improvement in global health status and functional scales and a negative change indicates an improvement in symptom and single item scales.

†Based on a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect and baseline as a covariate.

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

In order to determine if there was a treatment independent relationship between the FACIT-Fatigue quality of life instrument and intravascular hemolysis, an analysis was performed whereby the mean LDH level (through the 26 week study period) for each TRIUMPH patient was analyzed as a function of the patient's respective mean change in FACIT-Fatigue score from baseline (through the 26 week study period) (see Table 3). For this analysis, mean levels of LDH were divided into 4 groups that included: normal levels, 1-2 times the upper limit of normal (ULN), 2-10 times the upper limit of normal, and greater than 10 times the upper limit of normal. The analysis demonstrated that patients who maintained normal LDH levels throughout the study experienced significant improvements in fatigue when compared to patients who had incrementally higher levels of LDH throughout the study (p=0.0048). These data establish a clear link between increasing intravascular hemolysis as measured by LDH levels and decreased quality of life as measured by the FACIT-Fatigue quality of life instrument.

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

		Change of FACIT from Baseline		
Treatment Group	LDH Category	< 4	>= 4	P Value
Combined	Normal	4 (30.77%)	9 (69.23%)	.0048
	1 - 2 X ULN	16 (61.54%)	10 (38.46%)	
	2 - 10 X ULN	18 (72.00%)	7 (28.00%)	
	> 10 X ULN	16 (80.00%)	4 (20.00%)	

Safety

There were no deaths in the study. Serious adverse events (SAEs) were reported for 13 patients, of which 4 occurred in the eculizumab-treated cohort and 9 were in the placebo-treated cohort (see Table 4). All patients recovered without sequelae.

The most common AEs reported for eculizumab-treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infection. Headache and back pain occurred more commonly in the eculizumab-treatment group compared with the placebo group. However, the increase in headaches was limited to the first 2 weeks of therapy and was mild to moderate. There were no statistically significant differences in incidents rates between treatment groups for any AEs reported.

One episode of thrombosis (Budd-Chiari) occurred in a placebo-treated patient. There were no thromboses in eculizumab-treated patients.

Only one patient showed a detectable level of anti-eculizumab antibodies in the eculizumab-treated cohort; this response was weak (did not titrate), occurred at only one time point and did not result in a disruption of complement blockade.

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Table 4

Adverse Event Reporting

SERIOUS ADVERSE EVENTS*	**Placebo Patients n (percent)**	**Eculizumab Patients n (percent)**
Total	9 (20.5)	4 (9.3)
Eculizumab treatment emergent		
Exacerbation of PNH	3 (6.8)	1 (2.3)
Renal colic	0	1 (2.3)
Lumbar sacral disc prolapse	0	1 (2.3)
Alpha streptococcal bacteremia	0	1 (2.3)
Central line infection and UTI	1 (2.3)	0 (0)
Upper respiratory tract infection	1 (2.3)	0 (0)
Probable viral infection	1 (2.3)	0 (0)
Neutropenia	1 (2.3)	0 (0)
Cellulitis / folliculitis / neutropenia	1 (2.3)	0 (0)
Anemia and pyrexia	1 (2.3)	0 (0)
MOST FREQUENT ADVERSE EVENTS*†		
Headache§	12 (27.3)	19‡ (44.2)
Nasopharyngitis	8 (18.2)	10 (23.3)
Upper respiratory tract infection	10 (22.7)	6 (14)
Back pain	4 (9.1)	8 (18.6)
Nausea	5 (11.4)	7 (16.3)
Cough	4 (9.1)	5 (11.6)
Diarrhea	5 (11.4)	4 (9.3)
Arthralgia	5 (11.4)	3 (7.0)
Abdominal pain	5 (11.4)	2 (4.7)
Dizziness	5 (11.4)	2 (4.7)
Vomiting	5 (11.4)	2 (4.7)
Fatigue	1 (2.3)	5 (11.6)

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Viral infection	5 (11.4)	1 (2.3)

*By preferred terms

†Occurring in 10% or more of patients

‡Sixteen of 19 patients experienced headache within 48 hours of infusion

§Following the first 2 weeks of dosing, 20.9% of eculizumab- and 22.7% of placebo-treated patients experienced headache

DISCUSSION

Chronic intravascular hemolysis with periods of acute exacerbation are the classical manifestations of PNH, frequently resulting in anemia, the need for transfusions to sustain hemoglobin levels, and deterioration in quality of life. In the phase III pivotal TRIUMPH study, we examined the effect of terminal complement inhibition with eculizumab on hemoglobin levels and transfusion requirements in patients with PNH. Forty-nine percent of patients treated with eculizumab over the 6-month period demonstrated stabilization of hemoglobin in the absence of transfusions compared to no patients in the placebo arm of the trial. Over 50% of eculizumab-treated patients were transfusion independent during the entire study compared to none in the placebo arm, and the overall mean transfusion rate was reduced by 73%. Moreover, even in patients who did not achieve transfusion independence, eculizumab treatment was associated with a 44% reduction in the rate of transfusion (data now shown).

Lactate dehydrogenase, a biochemical marker of hemolysis in PNH,[9] was immediately and consistently decreased in all eculizumab-treated patients, while patients in the placebo cohort continued to hemolyze with levels of LDH exceeding 5 times the upper limit of the normal range in all patients at the study end. Levels of LDH were reduced into the normal range in approximately one-third of eculizumab-treated patients, while the remainder stabilized at a level just above the upper limit of normal suggesting residual low level hemolysis in some patients. Levels of haptoglobin, a more sensitive marker of the presence of cell free hemoglobin in the circulation, were undetectable in most patients. Low level hemolysis in a subset of eculizumab-treated patients is possibly due to an inherent decrease in survival of these cells or C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.[17]

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Before eculizumab treatment, hemoglobin levels in study patients were artificially maintained by frequent transfusion. Therefore, stabilization of hemoglobin levels with a concomitant cessation of or reduction in transfusions represents a net increase in endogenous hemoglobin levels. Our data suggest that resolution of hemolysis with eculizumab results in a new steady state hemoglobin level determined by a balance between the extent of the underlying bone marrow dysfunction, the number of PNH erythrocytes that are preserved by eculizumab therapy and the new level (if any) of transfusion requirement.

Patients with PNH generally experience markedly impaired quality of life characterized by fatigue, anemia, thrombosis, and pulmonary hypertension as well as smooth muscle dystonia including abdominal pain, dysphagia, and erectile dysfunction.[9,10,18] These symptoms have been attributed to both excessive intravascular hemolysis and downstream scavenging of nitric oxide by cell free hemoglobin in plasma. The reduction of intravascular hemolysis in eculizumab-treated patients in the current study was associated with significant improvements in the fatigue component of quality of life relative to placebo-treated patients as assessed via the FACIT-Fatigue instrument. Further, eculizumab therapy was associated with a median increase of 6.4 points over baseline values established before treatment. It has previously been demonstrated that an increase of 3 or more points from baseline represents a clinically important difference in this quality of life instrument.[19] Patients who received eculizumab also experienced a significant improvement in most domains of the EORTC QLQ-30 relative to the placebo-treated cohort including global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss, and insomnia. Improvement in the fatigue component of the EORTC QLQ-30 provides support for the improvement demonstrated in the FACIT-Fatigue instrument during eculizumab therapy. Importantly, these improvements in quality of life in the eculizumab-treated patients occurred despite similar levels of erythrocyte hemoglobin in the two treatment groups, further supporting the contribution of hemolysis per se, as opposed to anemia, in mediating the poor quality of life in PNH patients. Clinical assessment of additional life quality-related symptoms of PNH, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[20]

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Eculizumab was safe and well-tolerated. There were no deaths in the study and only a single thrombotic event which occurred in a placebo patient in a site (the hepatic veins) which is typical of the thrombosis in PNH. The relative brief duration of this study was not sufficient to address the relevant issue of a possible protection from thrombosis by terminal complement inhibition with eculizumab.

Adverse events were generally mild with headache occurring at increased frequency in the eculizumab-treated patients; however, this increased frequency did not persist following the first two doses of therapy. There were 4 SAEs in the eculizumab treatment group and 9 SAEs in the placebo group. There was no evidence of increased infection risk in eculizumab-treated patients during the study period. One eculizumab-treated patient showed a low level of anti-eculizumab antibodies at one time point during the study which did not persist and did not result in a disruption of complement blockade. There were no AEs associated with eculizumab withdrawal in the 2 eculizumab-treated patients who did not complete the trial. Additional safety assessments, as well as efficacy measures, are being examined in an ongoing multi-center, open-label Phase III safety trial of eculizumab (SHEPHERD) in approximately 95 patients with PNH.

Results from the current randomized, double-blind, placebo-controlled, global study show that terminal complement inhibition with eculizumab appears to be a safe and effective therapy for patients with the rare disorder PNH. Treatment with eculizumab reduced intravascular hemolysis, and stabilized hemoglobin levels despite a reduction of transfusions, to the point where most PNH patients were rendered transfusion independent. Substantial and clinically meaningful improvements in fatigue and other key quality of life parameters were also demonstrated. All of the 85 patients who completed the study elected to receive eculizumab in an open-label extension study and all currently remain on drug. The results of the TRIUMPH study indicate that terminal complement inhibition with eculizumab safely and effectively addresses an important consequence of the underlying genetic defect in PNH hematopoietic stem cells by providing a therapeutic replacement for the terminal complement inhibitor deficiency.

The present invention provides among other things treatment with an inhibitor of complement. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

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All publications and patents mentioned herein, including those items listed below, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

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SEQUENCES

SEQ ID NO: 1 – Eculizumab V_H

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPG
SGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFGSSPNWYF
DVWGQGTLVTVSSA

SEQ ID NO: 2 – Eculizumab Heavy chain

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPG
SGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFGSSPNWYF
DVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
RKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFN
WYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
SSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSL
SLSLGK

SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

SEQ ID NO: 5 – Eculizumab CDRH1

NYWIQ

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SEQ ID NO: 6 – Eculizumab CDRH2
EILPGSGSTEYTENFKD

SEQ ID NO: 7 – Eculizumab CDRH3
YFFGSSPNWYFDV

SEQ ID NO: 8 – Eculizumab CDRL1
GASENIYGALN

SEQ ID NO: 9 – Eculizumab CDRL2
GATNLAD

SEQ ID NO: 10 – Eculizumab CDRL3
QNVLNTPLT

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The invention furthermore comprises the following items:

1. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits complement or inhibits formation of C5b-9.

2. The method of item 1 wherein said quality of life is measured by a FACIT-Fatigue score.

3. The method of item 2 wherein the FACIT-Fatigue score increases by at least 3 points.

4. The method of item 2 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

5. The method of item 1 wherein said quality of life is measured by an EORTC QLQ-C30 score.

6. The method of item 5 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

7. The method of item 5 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

8. The method of item 7 wherein said aspect of quality of life is fatigue.

9. The method of item 1 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

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10. The method of item 1 wherein said compound is a steroid that suppresses complement.

11. The method of item 1 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

12. The method of item 11 wherein said compound is an antibody or an active antibody fragment.

13. The method of item 12 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

14. The method of item 12 wherein said antibody or active antibody fragment blocks C5 cleavage.

15. The method of item 12 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

16. The method of item 12 wherein said antibody is eculizumab.

17. The method of item 12 wherein said antibody or active antibody fragment is administered for at least 6 months.

18. The method of item 1 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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19. The method of item 1 wherein said patient is anemic

20. The method of item 19 wherein said patient remains anemic following treatment.

21. The method of item 19 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

22. The method of item 19 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

23. The method of item 19 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

24. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis.

25. The method of item 24 wherein said method results in a greater than 30% reduction in LDH in said patient.

26. The method of item 24 wherein said quality of life is measured by a FACIT-Fatigue score.

27. The method of item 26 wherein the FACIT-Fatigue score increases by at least 3 points.

28. The method of item 26 wherein the FACIT-Fatigue score increases by ≥ 4 points.

29. The method of item 24 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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30. The method of item 29 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

31. The method of item 29 wherein said aspect of the quality of life is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

32. The method of item 31 wherein said aspect of quality of life is fatigue.

33. The method of item 24 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

34. The method of item 24 wherein said compound is a steroid that suppresses complement.

35. The method of item 24 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

36. The method of item 35 wherein said compound is an antibody or an active antibody fragment.

37. The method of item 36 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

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38. The method of item 36 wherein said antibody or active antibody fragment blocks C5 cleavage.

39. The method of item 36 wherein said antibody or active antibody fragment is administered for at least 6 months.

40. The method of item 24 wherein said compound inhibits complement or inhibits the formation of C5b-9.

41. The method of item 40 wherein said compound is an antibody or active antibody fragment.

42. The method of item 41 wherein said antibody is eculizumab.

43. The method of item 24 wherein said patient has aplastic anemia or myelodysplastic syndrome.

44. A method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis, wherein said patient remains anemic.

45. The method of item 44 wherein said method results in a greater than 30% reduction in LDH in said patient.

46. The method of item 44 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

47. The method of item 44 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

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48. The method of item 44 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

49. The method of item 44 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

50. The method of item 44 wherein said quality of life is measured by a FACIT-Fatigue score.

51. The method of item 50 wherein the FACIT-Fatigue score increases by at least 3 points.

52. The method of item 50 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

53. The method of item 44 wherein said quality of life is measured by an EORTC QLQ-C30 score.

54. The method of item 53 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

55. The method of item 53 wherein said aspect of the quality of life is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

56. The method of item 55 wherein said aspect of quality of life is fatigue.

57. The method of item 44 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

58. The method of item 44 wherein said compound is a steroid that suppresses complement.

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59. The method of item 44 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

60. The method of item 59 wherein said compound is an antibody or an active antibody fragment.

61. The method of item 60 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

62. The method of item 60 wherein said antibody or active antibody fragment blocks C5 cleavage.

63. The method of item 60 wherein said antibody or active antibody fragment is administered for at least 6 months.

64. The method of item 44 wherein said compound inhibits complement or inhibits the formation of C5b-9.

65. The method of item 64 wherein said compound is an antibody or an active antibody fragment.

66. The method of item 65 wherein said antibody is eculizumab.

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67. The method of item 44 wherein said patient has aplastic anemia or myelodysplastic syndrome.

68. A method of prolonging the health-adjusted life expectancy of a patient comprising administering to said patient in need thereof a compound which inhibits formation of C5b-9.

69. The method of item 68 wherein said patient is anemic.

70. The method of item 69 wherein said patient remains anemic following treatment.

71. The method of item 68 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

72. The method of item 68 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

73. The method of item 68 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

74. The method of item 68 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

75. The method of item 68 wherein said health-adjusted life expectancy is measured according to a unit selected from the group consisting of Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, or Years of healthy life.

76. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one day.

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77. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least week.

78. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one month.

79. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one year.

80. The method of item 68 wherein said compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

81. The method of item 68 wherein said compound is a steroid that suppresses complement.

82. The method of item 68 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

83. The method of item 82 wherein said compound is an antibody or an active antibody fragment.

84. The method of item 83 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

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85. The method of item 83 wherein said antibody or active antibody fragment blocks C5 cleavage.

86. The method of item 83 wherein said antibody or active antibody fragment is administered for at least 6 months.

87. The method of item 68 wherein said compound inhibits complement or inhibits the formation of C5b-9.

88. The method of item 87 wherein said compound is an antibody or an active antibody fragment.

89. The method of item 88 wherein said antibody is eculizumab.

90. The method of item 68 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain comprising SEQ ID NOS: 8, 9 and 10.

2. The antibody of claim 1 comprising a light chain consisting of SEQ ID NO: 4.

3. A pharmaceutical composition comprising the antibody of claim 1 or 2.

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Europäisches Patentamt
European Patent Office
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Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	4744651
Application number	EP16195355.9
File No. to be used for priority declarations	EP16195355
Date of receipt	24 October 2016
Your reference	P2913 EP/3
Applicant	Alexion Pharmaceuticals, Inc.
Country	US
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Documents submitted	package-data.xml application-body.xml SPECEPO-1.pdf\P2913 EP_3 Specification.pdf (61 p.) OTHER-1.pdf\P2913 EP_3 Additional sheet.pdf (1 p.)	ep-request.xml ep-request.pdf (5 p.) SEQLTXT.txt\P2913 EP_3 Sequence listing.txt f1002-1.pdf (1 p.)

Submitted by	CN=Jasmin Soukenka 47563
Method of submission	Online
Date and time receipt generated	24 October 2016, 16:59 (CEST)
Message Digest	52:37:E5:FA:8E:E5:CE:B1:1E:88:CC:7C:2B:36:42:73:3A:24:67:35

Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/

ABSTRACT

Eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation, showed activity in a preliminary 12-week open-label trial in a small cohort of patients with paroxysmal nocturnal hemoglobinuria (PNH). The present study examined whether chronic eculizumab therapy could reduce intravascular hemolysis, stabilize hemoglobin levels, reduce transfusion requirements, and improve quality of life in a double-blind, randomized, placebo-controlled, multi-center global Phase III trial. It has been found that eculizumab stabilized hemoglobin levels, decreased the need for transfusions, and improved quality of life in PNH patients via reduced intravascular hemolysis. Chronic eculizumab treatment appears to be a safe and effective therapy for PNH.

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Patentanwälte Rechtsanwälte mbB

EPO - Munich
50
20. Dez. 2016

VOSSIUS & PARTNER · POB 86 07 67 · 81634 München · Germany

European Patent Office

80298 M U N I C H

1. EP-Patent Application 07 75 3249.7
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP

2. EP-Patent 2359834
   (EP 11 00 1632.6)
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/1

3. EP-Patent Application 16 17 2335.8
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/2

4. EP-Patent Application 16 19 5355.9
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/3

5. EP-Patent 2380907
   (EP 11 00 5615.7)
   Alexion Pharmaceuticals, Inc.
   Our Ref.: R1249 EP/1

6. EP-Patent Application 16 20 1244.7
   Alexion Pharmaceuticals, Inc.
   Our Ref.: R1249 EP/3

München, December 20, 2016
WA/FLG/SOJ

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5) European Patent Attorney
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8) Abogada (Spain)
9) mit Zweigstelle in Berlin
10) Solicitor (England & Wales)
11) kein European Trademark Attorney
12) auch Patentanwalt - Deutschland

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Switzerland: HR Firmennummer CH-270.9.001.271-9

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It is respectfully requested that the change of address be recorded in the European Patent Register and that EPO Form 2544 be issued promptly.

Dr. Joachim Wachenfeld
European Patent Attorney

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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

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Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
02.01.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

C o m m u n i c a t i o n

concerning the registration of amendments relating to

☐ a transfer (R. 22 and 85 EPC)

☑ entries pertaining to the applicant / the proprietor (R. 143(1)(f) EPC)

As requested, the entries pertaining to the applicant of the above-mentioned European patent application / to the proprietor of the above-mentioned European patent have been amended to the following:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SI SK TR
Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510/US

The registration of the changes has taken effect on 20.12.16.

In the case of a published application / a patent, the change will be recorded in the Register of European Patents and published in the European Patent Bulletin (Section I.12 / II.12).

Your attention is drawn to the fact that, in the case of the registration of a transfer, any automatic debit order only ceases to be effective from the date of its express revocation (cf. point 14(c) of the Arrangements for the automatic debiting procedure, supplementary publication 3 - OJ EPO 2015).

Receiving Section / For the Examining Division / For the Opposition Division / For the Legal Division *)

Europäisches Patentamt • European Patent Office • Office européen des brevets

*) See note.

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Note
This communication is issued by/for the department with whom responsibility lies. The Legal Division is responsible for the registration of transfers, changes of name (Articles 71, 72 and 74 EPC and Rules 22 and 85 EPC) as well as for the rectification of the designation of the inventor (Rule 21 EPC) (see Decision of the President of the EPO, OJ EPO 2013, 600). In all other cases, the Receiving Section, the Examining Division or the Opposition Division is responsible, depending on the stage in proceedings.

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Payment of fees and expenses

European Patent Office
Treasury and Accounting
80298 München
Germany
Fax +49(0)89 2399-4465

Alexion Pharmaceuticals, Inc.

Please complete in typescript only

Name of payer

VOSSIUS & PARTNER (No. 31)
Patentanwälte Rechtsanwälte mbB

Address

Siebertstraße 3
81675 München
Germany

Payer's reference

P2913 EP/3

Mode of payment

☐ Bank transfer to Commerzbank AG[1]
IBAN: DE20 7008 0000 0333 8800 00
BIC: DRESDEFF700

EPO - Munich
100
16. Feb. 2017

☒ Debit from deposit account with the EPO is requested[2]

Deposit account No. 2800.0321

Patent application/patent No. (please use a separate form for each application)

EP 16 19 5355.9

PCT

Code		Amount/EUR
001	Filing fee – EP direct[3]	
501	Additional fee (more than 35 pages)	
020	Filing fee – entry EP phase[3] (Rule 159(1)(c) EPC)	
520	Additional fee (more than 35 pages) - entry EP phase	
	Additional fee for divisional applications (Rule 38(4)EPC)[9]	
002	Fee for a European search	
055	Additional copy of docs cited in search report	
015	Claims fee(s) (Rules 45(1), 162(1)EPC)[5]	
005	Designation fee(s)[4]	
006	Examination fee	
122	Fee for further processing (non-fee related cases)	
123	Fee for further processing (late payment of a fee)	
007	Fee for grant and printing or fee for grant incl. fee for publication[6]	
008	Additional printing fee for 36th and each subsequent page[7]	
016	Claims fee according to Rule 71(4) EPC[5]	
121	Fee for further processing (late performance of acts R. 71(3))	
022	Registering of transfer	

Code		Amount/EUR
010	Opposition fee	
011	Fee for appeal	
029	Certified copy of application; priority document	
033	Renewal fee for the 3rd year	470,00
034	Renewal fee for the 4th year	585,00
035	Renewal fee for the 5th year	820,00
036	Renewal fee for the 6th year	1.050,00
	Validation for[8]	
037	Renewal fee for the 7th year	1.165,00
038	Renewal fee for the 8th year	1.280,00
039	Renewal fee for the 9th year	1.395,00
	Extension for[8]	
040	Renewal fee for the 10th year	1.575,00

Total **8.340,00**

Notes 1 - 9 see overleaf.

Signature (handwritten / no block letters)

Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date

16. FEB. 2017

München, February 24, 2017, WA/FLG/SOJ

EPO 1010 11.15

Europäisches Patentamt
European Patent Office
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Application No.: 16195355.9

The search has started on 03.03.17 (see Notice from the European Patent Office dated 03/2013, OJ EPO 2013, 153).

Receiving Section

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 16 19 5355

TITLE: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLICANT: Alexion Pharmaceuticals, Inc.

IPC CLASSIFICATION: A61K31/7105, A61K38/00, A61K39/395, A61P7/06, C07K16/18, A61K9/00

EXAMINER: Collura, Alessandra

CONSULTED DATABASES: WPI, EMBASE, MEDLINE, REG, CA

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
antibody that binds to C5 having the indicated heavy and light chain

EPO FORM P04A42

Europäisches Patentamt
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EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01) * the whole document * * paragraphs [0036], [0037], [0082] * -----	1-3	INV. A61K31/7105 A61K38/00 A61K39/395 A61P7/06
X	HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)", BRITISH JOURNAL OF HAEMATOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758, ISSN: 0007-1048 * the whole document * -----	1-3	C07K16/18 A61K9/00
X,D	HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA", NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688 * abstract * -----	1-3	TECHNICAL FIELDS SEARCHED (IPC) A61K

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

1

EPO FORM 1503 03.82 (P04C01)

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Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HARDING J: "Eculizumab", DRUGS OF THE FUTURE, PROUS SCIENCE, ES, vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000, ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330 * the whole document * * page 674, left-hand column, paragraph 3 * -----	1-3	
X	HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426, ISSN: 1543-0790 * the whole document * -----	1-3	
X	DATABASE REGISTRY [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14), XP002767828, retrieved from STN accession no. 219685-50-4 Database accession no. 219685-50-4 * abstract * -----	1,2	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

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Page 122

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 19 5355

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

06-03-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005191298 A1	01-09-2005	AU 2005209854 A1	18-08-2005
		CA 2554632 A1	18-08-2005
		CN 1938047 A	28-03-2007
		CN 103446584 A	18-12-2013
		CN 103977402 A	13-08-2014
		CN 103977403 A	13-08-2014
		CY 1113190 T1	13-04-2016
		DK 1720571 T3	03-09-2012
		EP 1720571 A2	15-11-2006
		EP 2520311 A2	07-11-2012
		EP 2522361 A1	14-11-2012
		EP 2522362 A1	14-11-2012
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		EP 2522364 A1	14-11-2012
		EP 2910253 A1	26-08-2015
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		HK 1094950 A1	01-02-2013
		JP 5873047 B2	01-03-2016
		JP 2007520494 A	26-07-2007
		JP 2011126917 A	30-06-2011
		JP 2013213044 A	17-10-2013
		JP 2015083611 A	30-04-2015
		KR 20070009996 A	19-01-2007
		NZ 549328 A	24-12-2010
		PL 1720571 T3	30-11-2012
		PT 1720571 E	29-08-2012
		SI 1720571 T1	30-10-2012
		US 2005169921 A1	04-08-2005
		US 2005191298 A1	01-09-2005
		US 2010068202 A1	18-03-2010
		US 2011086040 A1	14-04-2011
		US 2012308559 A1	06-12-2012
		WO 2005074607 A2	18-08-2005

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

The examination is being carried out on the **following application documents**

Description, Pages

1-55 as originally filed

Sequence listings, SEQ ID NO

1-10 as originally filed

Claims, Numbers

1-3 as originally filed

Drawings, Sheets

1/4-4/4 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01)

D2 HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)",
BRITISH JOURNAL OF HAEMATOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB,
vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758,
ISSN: 0007-1048

D3 HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA",
NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US,
vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948,
ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688

D4 HARDING J: "Eculizumab",
DRUGS OF THE FUTURE, PROUS SCIENCE, ES,
vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000,
ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330

D5 HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria",
CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US,
vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426,
ISSN: 1543-0790

D6 DATABASE REGISTRY [Online]
CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14),
retrieved from STN accession no. 219685-50-4
Database accession no. 219685-50-4

Fro what concerns the most relevant passages of the above-mentioned documents, please see citations in the European Search Report, unless otherwise indicated.

1. Article 76(1) EPC

The present application does not meet the requirements of Article 76(1) EPC, because it relates to subject-matter which extends beyond the parent application as originally filed.

The applicant indicated page 5, last paragraph as basis for the claims of the application.

However, said paragraph puts into relation sequence 2 with sequence 4 and not with the specific sequences 8, 9 and 10. These last sequences are explicitly mentioned only in relation with specific sequences 5, 6 or 7.

2. Novelty

The present application does not meet the requirements of Article 54 EPC, because the subject-matter of the claims 1-3 is not novel.

The attention of the applicant is drawn to the fact that the present claims are directed to a product and are not limited in scope by any other features than those relating to the product itself.

The sequences indicated in the claims are told to be the sequences of the heavy and light chains of eculizuman or part of them.

Therefore, any document disclosing eculizumab published before the filing date of the present application obviously destroys the novelty of the present claims.

All documents cited in the search report refer to eculizumab and to the fact that it is an antibody which binds C5. D1-D5 also describe pharmaceutical compositions of the compound.

3. Inventive step

The present application does not meet the requirements of Article 56 EPC, because the subject-matter of the claims 1-3 is not inventive.

As a matter of fact, claims which are not novel cannot be considered to involve any exercise of an inventive activity.

4. Procedure

The applicant is invited to file new claims which take account of the above comments.

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 123(2) EPC, the applicant should clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see Guidelines E-II, 1).

The attention of the applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed (Article 123(2) EPC).

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European Patent Office
80298 MUNICH
GERMANY

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
17.03.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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Page 127

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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date: 05.04.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466

Applicant/Proprietor: Alexion Pharmaceuticals, Inc.

Refund of fees

The following fee(s) was (were) paid in respect of the application 16195355.9:

Fee	Reference	Voucher No	Date	Currency	Amount
Search fee	002	01354109	24.11.16	EUR	1 300,00

The fee(s) was (were) paid by Vossius & Partner.

According to the present state of the file the refund will be made by:

CREDITING THE DEPOSIT ACCOUNT 28000321 Vossius & Partner.

Amount refundable:	Reference	Currency	Amount	Voucher No
	002	EUR	1.300,00	00739430

Reason for refund: Full use of earlier European search report with written opinion drawn up by the EPO (100 % of fee paid for current search refunded).

The Authorising Officer
Adams
(49)(89)23992668

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Page 128

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
20.04.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Notification of European publication number and information on the application of Article 67(3) EPC

You are hereby informed that the technical preparations for the publication of the above-mentioned European patent application have been completed.

The application will be published on 17.05.17 with the European search report.
The publication number is: 3167888.
The publication will be mentioned in European Patent Bulletin number 2017/20.
(**http://bulletin.european-patent-office.org**).

Provisional protection under Article 67(1) and (2) EPC in the individual contracting states becomes effective when the conditions referred to in Article 67(3) EPC have been fulfilled. Provisional protection in the individual extension and validation states is governed by the national laws of the countries concerned. For more details, see Chapter III of the EPO brochure "National Law relating to the EPC" (**www.epo.org/law-practice/legal-texts/national-law.html**), which also contains information about the extension and validation system. Further information can also be found in the Official Journal of the European Patent Office.

The title of the invention in the three official languages of the European Patent Office is worded as follows:

BEHANDLUNG VON PATIENTEN MIT PAROXYSMALER NÄCHTLICHER HÄMOGLOBINURIE MIT EINEM KOMPLEMENTINHIBITOR
TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
TRAITEMENT DE PATIENTS SOUFFRANT D'HÉMOGLOBINURIE PAROXYSTIQUE NOCTURNE PAR UN INHIBITEUR DE COMPLÉMENT

In all future communications to the EPO, please quote the application number as indicated above, i.e. including the final four figures (which identify the Directorate responsible for the subsequent procedure).

Remark
The publication can be downloaded, free of charge, from the EPO publication server **https://data.epo.org/publication-server/** or can be ordered from the Vienna sub-office upon payment of a fee (see Decision of the President of the EPO dated 12 July 2007 concerning the form of publication of European patent applications, European search reports and European patent specifications, Special edition No. 3, OJ EPO 2007, 97).

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Note concerning renewal fees
Under Article 86 EPC renewal fees shall be paid to the EPO for the European patent application. These fees shall be due in respect of the third and each subsequent year, calculated from the date of filing. Due account should be taken that the EPO will not send invoices or reminders to pay any fees in due time.

Accelerated prosecution of European patent applications
Applicants wishing to accelerate proceedings before the EPO may file a request according to the programme for accelerated prosecution of European patent applications - "PACE" (OJ EPO 2010, 352). EPO Form 1005 is available on the EPO website (www.epo.org) for this purpose.

Receiving Section



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European Patent Office
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80298 MUNICH
GERMANY

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
22.05.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication pursuant to Rule 69 EPC - reminder concerning payment of the designation fee (Art. 79(2) EPC) and of the examination fee (Art. 94(1) EPC) - and invitation pursuant to Rule 70a(1) EPC

The date on which the European Patent Bulletin mentions the publication of the European search report for the above-mentioned European patent application is: 17.05.17.

Your attention is drawn to Article 79(2) and Rule 39(1) EPC as well as Article 94(1), Rule 70(1) and Rule 70a(1) EPC, according to which within **six months** of the above-mentioned publication date of the search report

- the designation fee must be paid,
- the request for examination must be filed and the examination fee must be paid,
- comments on the objections raised in the opinion accompanying the European search report and/or any amendments to the description, claims and drawings correcting any deficiencies noted in the opinion must be filed (R. 70a(1), 137(2) EPC; Guidelines for Examination in the EPO, B-XI, 8).

1. Payment of the designation fee and of the examination fee

The current designation fee for one or more Contracting States designated is: EUR 585,00

The current examination fee is: EUR 1635,00

If the written request for examination is filed in an admissible non-EPO language in accordance with Article 14(4) EPC, a reduction of the examination fee according to Rule 6(3) EPC applies.

For European patent applications filed on or after 01.04.2014, the reduction applies only if the applicant(s) has (have) declared to be an entity or a natural person within the meaning of Rule 6(4) EPC (Decision of the Administrative Council of 13 December 2013, OJ EPO 2014, A4).

If the designation fee and the examination fee are not paid within the period laid down in Rule 39(1) and Rule 70(1) EPC, the application **will be deemed to be withdrawn** (R. 39(2) and Art. 94(2) EPC).

Any extension or validation fees are also payable within the above-mentioned period.

Registered letter

2. Comments on the opinion accompanying the European search report and filing of amendments

Should the reply to the invitation pursuant to Rule 70a(1) EPC be filed in an admissible non-EPO language, a translation is to be submitted within one month of its filing (R. 6(2) EPC).

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the Examining Division requesting that you correct this deficiency (R. 137(4) EPC).

Should you not comply with this invitation within the time limit, the application **will be deemed to be withdrawn** in accordance with Rule 70a(3) EPC.

Note to users of the automatic debiting procedure
Unless the EPO receives prior instructions to the contrary, the examination fee and the designation fee will be debited on the last day of the period for payment. For further details see the Arrangements for the automatic debiting procedure, supplementary publication 3 - OJ EPO 2015.

Important information concerning fee amounts
Following any amendment to the Rules relating to Fees, the amount(s) mentioned in this communication may be different from the amount(s) **actually due on the date of payment.** The latest version of the Schedule of fees and expenses, published as a Supplement to the Official Journal of the EPO, is also available on the EPO website (**www.epo.org**) and can be found under **www.epo.org/schedule-of-fees**, which allows the viewing, downloading and searching for individual fee amounts, both current and previous.
Please note that procedural fees are usually adjusted every two years, on even years, with effect from 1 April.

Payments by cheque delivered or sent direct to the EPO are no longer accepted as from 1 April 2008 (see OJ EPO 2007, 626).

Note concerning renewal fees
Under Article 86 EPC renewal fees shall be paid to the EPO for the European patent application. These fees shall be due in respect of the third and each subsequent year, calculated from the date of filing. Due account should be taken that the EPO will not send invoices or reminders to pay any fees in due time.

Receiving Section

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SEQUENCES

SEQ ID NO: 1 – Eculizumab V_H

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPG
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DVWGQGTLVTVSSA

SEQ ID NO: 2 – Eculizumab Heavy chain

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SEQ ID NO: 3 – Eculizumab V_L

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SEQ ID NO: 4 – Eculizumab Light chain

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NYWIQ

SEQUENCE LISTING

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<150> 60/783,070
<151> 2006-03-15

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35 40 45
Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
50 55 60
Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
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35 40 45
Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
50 55 60
Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
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Ala Arg Tyr Phe Phe Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val Trp
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        195                 200                 205
His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys
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Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
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    290                 295                 300
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Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
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Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn Thr Pro Leu
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
            100                 105
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<210> 4
<211> 214
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polypeptide

<400> 4
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Gly Ala Ser Glu Asn Ile Tyr Gly Ala
20 25 30
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn Thr Pro Leu
85 90 95
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
100 105 110
Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
115 120 125
Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
130 135 140
Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145 150 155 160
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
165 170 175
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180 185 190
Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
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Phe Asn Arg Gly Glu Cys
210

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<223> Description of Artificial Sequence: Synthetic peptide

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1 5

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EPO - Munich
100
10. Juli 2017

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB

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European Patent Office

80298 M U N I C H

EP 16 19 5355.9 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/3

München, July 10, 2017
WA/FLG/SOJ

I. COMMUNICATION PURSUANT TO RULE 69 EPC

Payment of the examination fee and the designation fee for all contracting states is effected with the enclosed sheet of Payment of fees and expenses (EPO Form 1010). Examination is herewith requested.

II. REPLY TO THE EESR PURSUANT TO RULE 70a(1) EPC

This is in reply to the extended European search report issued in the above-mentioned case on March 17, 2017.

1. INADMISSIBLE BROADENING (Article 76(1) EPC)

1.1. The Examiner is of the view that the subject-matter of claim 1 cannot be clearly and unambiguously derived from the parent application as filed.

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Switzerland: HR Firmennummer CH-270.9.001.271-9

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1.2. Claim 1 is directed to an antibody comprising the complete heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10).

1.3. The basis for pending claim 1 in the parent application as filed can be found on page 9, last paragraph of the parent application as filed which reads:

> "In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. [...] In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ ID NO: 4."

On pages 44 and 45 of the apparent application as filed SEQ ID NOs NOs 1 to 10 are shown and it is described that all of them characterize the antibody eculizumab. Hence, in order arrive at claim 1 it is required to combine features of a broader embodiment and a narrower embodiment both characterizing the structure of eculizumab. As the Examining Division is aware, this is generally seen in the EPO as resulting in a so-called "intermediate generalisation".

In T 461/05 the Board of Appeal ruled that an intermediate generalisation has to be held distinct from a simple generalisation, since in the former case a definition of the invention in general terms forms part of the original disclosure. In accordance with the pertinent case law an intermediate generalisation can be accepted under Articles 123(2) and 76(1) EPC in the absence of any clearly recognisable functional or structural relationship among the features of the specific combination (see, for example, T 1067/97 and T 25/03), or if the extracted feature is not inextricably linked with those features (see T 714/00).

Moreover, the general "gold standard" (see G 2/10) for assessing compliance with Articles 123(2) and 76(1) EPC is that amendments to the claims can only be made within the limits of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed.

1.4. In the present case the skilled person could be an immunologist. The structure of an antibody is part of the common general knowledge of an immunologist. As the Examining Division is aware, an antibody (monomer) is a "Y"-shaped molecular structure that consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Three variable loops each on the light and heavy chains are responsible for binding to the antigen. These loops are referred to as the complementarity determining regions (CDRs).

Thus, the skilled person knows that an antibody is composed of two different sequences, the light and heavy chain. These two chains are connected by disulfide bonds. The skilled person also knows that while the complete chains exhibit binding specificity for a particular antigen, the binding specificity is determined by the unique amino acid sequences found in the three hypervariable complementarity determining regions known as the CDRs.

Hence, the skilled person on the basis of the above-quoted last paragraph on page 9 of the parent application as filed arrives at the antibody of claim 1 comprising the heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10) without the requirement of extracting a feature being inextricably linked with other features. In line with T 714/00 this constitutes an allowable intermediate generalisation. It is of further note that since the light and heavy chain are separate there is no functional or structural relationship among these sequences that distracts a skilled person from directly and unambiguously deriving the antibody of claim 1. Thus, also in line with T 1067/97 and T 25/03 claim 1 constitutes an allowable intermediate generalisation.

In summary, it is our firm position that the claims find a clear and unambiguous basis in the parent application as filed as well as the application as filed.

2. NOVELTY AND INVENTIVE STEP (Articles 54 and 56 EPC)

2.1. The Examiner objects to the novelty of claims 1 to 3 in view of documents D1 to D5. The Examiner states that each of these documents describes eculizumab.

In view of the alleged lack of novelty the Examiner also contests inventive step of claims 1 to 3.

2.2. Because the use of eculizumab for the treatment of PNH is described in D1 to D5, the Examiner apparently assumes that eculizumab must have been publically available before the filing date of the present application.

We respectfully note that this assumption is not correct because the mere naming of a drug such as eculizumab in a publication describing a confidential clinical trial does not place the biochemical structure in the prior art or in public use. As is evident from the enclosed declaration from the corresponding US proceedings by the inventor of the present application, founder and CEO of the Applicant Alexion Pharmaceuticals, Inc. Dr. Leonard Bell, prior to the March 15, 2007 effective filing date of the present application, the complete structure of eculizumab was not disclosed in the prior art. Also, eculizumab was never provided to a member of the public absent strict confidentiality and storage restrictions before March 15, 2007, as described in the enclosed declaration from Dr. Laural Boone that was submitted in the corresponding US proceedings..

Moreover, D1 to D5 fail to teach or suggest any part of the sequence of eculizumab, and in particular the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab (including the CH1-hinge-CH2-CH3 regions) of SEQ ID NO: 2. This engineered full heavy chain confers important and unexpected functional effects to eculizumab, as described in the declaration of Dr. Bell.

In more detail and as evidenced by the sequence alignment within the declaration, the heavy chain constant regions of eculizumab and a naturally-occurring IgG4 heavy chain differ in fourteen amino acid residues (i.e., four different residues in the CH1 domain, six different residues in the hinge region, and four different residues in the CH2 domain). Furthermore and as also discussed in the accompanying declaration, these structural differences confer eculizumab with advantageous functional features, including decreased effector function, reduced immunogenicity and increased half-life.

In sum, the complete structure of eculizumab as currently claimed – being an antibody characterized by the heavy of SEQ ID NOs 2 and the light chain CDRs of SEQ ID NOs 8, 9 and 10 – was not disclosed or otherwise made available to the public prior to the March 15, 2007 effective filing date of the present application. Therefore, prior to the effective filing date of the present application the complete structure of eculizumab as currently claimed in the present application was neither taught nor suggested in the cited prior art; nor was it obtainable by way of public availability.

We therefore submit that the new claims are novel over D1 to D5. In view of the above-discussed unexpected and advantageous technical properties being associated with the complete structure of eculizumab, we submit that the claims are also inventive.

3. CORRECTION OF AN OBVIOUS ERROR (Rule 139 EPC)

Enclosed herewith is corrected page 44 of the application as filed and a corrected sequence listing, wherein an obvious error in the sense of Rule 139 EPC has been corrected in SEQ ID NOs 3 and 4. In more detail, the light chain of eculizumab as shown in SEQ ID NO: 4 as well as the corresponding shorter V_L sequence of eculizumab as shown in SEQ ID NO: 3 as originally filed erroneously comprise the N-terminal 22-amino acids long leader peptide which cannot be found in the mature light chain as comprised with the mature antibody. In the corrected SEQ ID NOs 3 and 4 the leader peptide has been removed.

We submit that the requested correction of SEQ ID NOs 3 and 4 complies with the prerequisites of Rule 139 EPC, because the skilled person in the field of antibodies immediately recognizes

(I) that SEQ ID NOs 3 and 4 are not correct, and

(II) that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22-amino acids long leader peptide; see Guidelines for Examination in the EPO, Part H – Chapter VI-2.2.

In this respect we refer the Examining Division to the enclosed declaration from the technical expert in the field of antibodies Dr. Bruce Andrien (Director, Early Assay Development, Alexion Pharmaceuticals, Inc.).

Regarding prerequisite (I), it can be taken from section 8 of the declaration that the skilled person immediately recognizes that SEQ ID NO: 4 is not correct, because (i) SEQ ID NO: 4 starts with methionine (M), (ii) M is followed by a stretch of 21 amino acids being mostly hydrophobic, and (iii) SEQ ID NO: 4 is with 236 amino acids considerable longer than typical mature antibody light chains of 211 to 217 amino acids. It was common textbook knowledge at the priority date in 2006 that the start codon of the genetic code (ATG) codes for the amino acid M and therefore most unprocessed protein sequences start with M. It

was also commonly known that antibody light chains originally contain a leader peptide beginning with a methionine and followed by a hydrophobic core section (known as leader or signal petide) that is removed during protein processing and maturation. As regards the length of 211 to 217 amino acids of mature antibody light chains the declaration references the textbook Janeway et al., Immunology, 5th ed. (2001). The contents of textbook as well as handbooks, monographs, encyclopaedias and reference books establish the common general knowledge of the skilled person; see 8th ed. Of the Case Law book, section I.C.2.8.1, second paragraph. Hence, it is immediately recognisable for the skilled person, using common general knowledge, from the originally-filed application documents taken by themselves that SEQ ID NOs 3 and 4 are erroneous because they contain the signal peptide that is not present in the mature protein.

Prerequisite (II) is considered to be met if the correction is within the limits of what a skilled person derives directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the originally-filed application documents. As can be taken from sections 9 to 12 of the declaration of Dr. Andrien, the skilled person immediately recognizes that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22 amino acids long leader peptide, when considering the common general knowledge of the skilled person as being evidenced by the contents of the Kabat database in the last version as a textbook. The Kabat Database was first published as textbook in 1976 and the last edition in textbook form was published in 1991. Since then the Kabat database is accessible online. Since its establishment until today the Kabat database is extensively and foremost used by people skilled in the field of antibodies in order to derive structural and functional information on primary sequences of antibodies. As of its establishment, it was the most complete compilation of data in the antibody field. In the antibody field it had an equally high importance as the Maniatis and later Sambrook, "Molecular Cloning" textbook in the field of molecular cloning. It follows that the contents of the Kabat database and in particular the early contents as published in the format of a textbook are to be considered common general knowledge of the skilled person in the field of antibodies. Hence, it was common general knowledge of the skilled person at the priority date in 2006 that (i) leader peptides of kappa antibody light chains are usually 22 amino acids long

and start with the amino acids "MDM" (see section 11 of the declaration), and (ii) that the mature form of kappa antibody light chains usually starts with the amino acids "DIQM" (see section 11 of the declaration), noting that also the leader peptide of eculizumab is 22 amino acids long and starts with the amino acids "MDM" and that also the mature antibody light chain of eculizumab starts with the amino acids "DIQM".

In summary, the requested correction of the antibody light chain of SEQ ID NO: 4 and consequently also the corresponding V_L sequence of SEQ ID NO: 3 are within the limits of what a skilled person can derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the documents as filed; see also G 3/89 and G 11/91. We therefore submit that our request for the correction of SEQ ID NOs 3 and 4 under Rule 139 EPC is fully justified.

4. REQUESTS

With the above amendments and explanations it is submitted that the applicant has met the requirements as set forth in the EESR. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

If, however, the Examining Division does not agree to the above, it is requested that either a Communication pursuant to Article 94(3) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosures:
Bell Declaration
Andrien Declaration (including Exhibits)
Boone Declaration
EPO Form 1010
Corrected Page 44
Corrected sequence listing (also on CD-ROM)

Samsung Bioepis Exhibit No. 1028
Page 144

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Payment of fees and expenses

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10. Juli 2017

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EP 16 19 5355.9-1466

PCT

Code		Amount/EUR
001	Filing fee – EP direct[3]	
501	Additional fee (more than 35 pages)	
020	Filing fee – entry EP phase[3] (Rule 159(1)(c) EPC)	
520	Additional fee (more than 35 pages) - entry EP phase	
	Additional fee for divisional applications (Rule 38(4)EPC)[9]	
002	Fee for a European search	
055	Additional copy of docs cited in search report	
015	Claims fee(s) (Rules 45(1), 162(1)EPC)[5]	
005	Designation fee(s)[4]	585,00
006	Examination fee	1.635,00
122	Fee for further processing (non-fee related cases)	
123	Fee for further processing (late payment of a fee)	
007	Fee for grant and printing or fee for grant incl. fee for publication[6]	
008	Additional printing fee for 36th and each subsequent page[7]	
016	Claims fee according to Rule 71(4) EPC[5]	
121	Fee for further processing (late performance of acts R. 71(3))	
022	Registering of transfer	

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010	Opposition fee	
011	Fee for appeal	
029	Certified copy of application; priority document	
033	Renewal fee for the 3rd year	
034	Renewal fee for the 4th year	
035	Renewal fee for the 5th year	
036	Renewal fee for the 6th year	
	Validation for[8]	
	Extension for[8]	

Notes 1 - 9 see overleaf.

Total **2.220,00**

Signature (handwritten / no block letters)

Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date

München, July 10, 2017, WA/FLG/SOJ

EPO 1010 11.15

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☐ (.....)^{n} Prospect(s)/Brochure(s)
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Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

Exhibit A

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CAREER EXPERIENCE:

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Early Assay Development; July 2015 - Present.

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- Developed processes to support Research's ever expanding needs including testing of proteins within the following areas: Biacore, HPLC, LOC, IEF, Mass Spectrometry, and biological assays.
- Lead the Process Development and Analytical Sciences Quality Improvement Team
- Lead the Process Development and Analytical Sciences Bi-Paratopic team
- Supported four diligences teams
- Supported three early research teams
- Provide response to regulatory agency questions

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Analytical Sciences; July 2014 – July 2015.

GMP Compliance responsibilities

- Provide analytical support of CMC submissions, address comments from regulatory agencies regarding test methods and validation
- Direct and coordinate the transfer of release and stability test methods into Quality Control or to contract laboratories worldwide to support release testing of commercial drug product
- Provide support and troubleshooting to Quality Control groups for analytical test methods. Support includes assisting in assay invalids and/or OOS investigations, aberrant trends or special studies, etc.
- Manage, delegate, and prioritize GMP release and stability testing for all new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Direct formulation development activities, oversee design, execution, and analysis of formulation development studies.
- Manage qualification and validation of analytical methods for early stage clinical products in addition to existing commercial products.
- Manage in-process testing and coordinate outsource testing for new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Coordinate quarterly review meetings and provide updates, and generate reports for early stage (Phase 1 and Phase 2) clinical products.
- Generate or revise specifications for early stage (Phase 1 and Phase 2) clinical products
- Maintain appropriate compliance within area of responsibility with regards to current SOPs, validation / qualifications, and training. Represent the area functions as required during audits by external agencies and auditors, as well as by internal auditors
- Establish systems within area of responsibility to ensure compliance, good lab practices and efficiency

Development responsibilities

- Direct the development, improvement, qualification, and validation of analytical testing of proteins. Tests within area are: HPLC, SDS-PAGE, Western blots, IEF, ELISAs, biological assays and protein determinations.
- Generate and oversee product comparability protocols for early stage (Phase 1 and Phase 2) clinical products.
- Oversee the analytical support of cell culture and purification development groups in support of new clone or media optimizations and improvements to purification processes.
- Work with Purification Development to plan and coordinate formulation development studies, including generating and executing formulation stability protocols, compiling data, trending and presenting to all facets of the organization.

Other Responsibilities:

- Train, coach, and mentor Analytical Sciences laboratory personnel by working with employees to develop performance objectives and to monitor performance against the objectives.
- Interact with Alexion Pharmaceuticals personnel within Development, and other departments as required to meet objectives; interact with Alexion Pharmaceuticals business partners and technical representatives as required to meet objectives
- Represent Analytical services on Task Force or Planning Committees as assigned

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Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Protein Characterization; Sep. 2001 – Jul. 2014.

Manage the Protein Characterization group for characterizing proteins to support IND and BLA filings along with drug product understanding for FDA, EMEA, TGA, PMDA, and Health Canada. These assays include SDS-Page, vIEF, hIEF, 2D-Page, enzyme digestions, HPLC-UV (peptide maps, oligosaccharide, monosaccharide, sialic acid), CD, fluorometry, ligand/antigen kinetics by surface plasma resonance, N-terminal, flow field fractionation multi angel light scattering, ion mobility, mass spectrometry (small molecule contamination identification, intact molecular weight, peptide maps by µLC/nLC ion trap/TOF/Q-TOF, MALDI peptide mass fingerprints, disulfide mapping, identification of unknown proteins, glycan characterization, peptide confirmation by LC-MS/MS and MALDI-PSD, and in-house bioinformatics and proprietary Alexion database). Method robustness and method transfer. Review, write and oversee SOP's, GTR's, and protocols.

Managed more than $1,000,000 budget/year along with FTE's including Associate Directors, Ph.D., Masters, and Bachelors level as direct reports at Alexion.

Managed the staff meeting commutations between VP and Fermentation, Purification, Analytical Sciences, and Protein Characterization Groups.

Managed CMC documentation personal, review and write CMC sections for IND, NDA, BLA, updates and responses for drug product and drug substance for FDA, EMEA and other countries.

Support Alexion's Research, Fermentation, Purification, Quality Control/Quality Assurance and Clinical with techniques available in Protein Characterizations core facility.

Analytica of Branford, Inc., Branford, Connecticut; Senior Research Scientist; 1991 - 2001.

Conducted research and development on time-of-flight mass spectrometers, ion guides, ion optics, electrospray, nanoelectrospray and atmospheric pressure chemical ionization ion sources. Developed automation techniques using flow injection analysis and liquid chromatography for proteins, peptides, and small molecules. Generated data to support marketing, client needs, competitive demos, product development, research, and conference presentations. Author of patents, grants, manuals for various hardware, conference presentations, articles, and internal reports.

Principle Investigator on phase I and phase II NIH grants. Project manager of nanospray, dual electrospray, APCI, and parts of a TOF-MS project, taking these from the initial research and development phase though prototype design and finally to production and qualification testing. Responsible for installations, client training, and trouble shooting for electrospray, nanospray, dual electrospray, APCI, and TOF-MS products.

Conducted computer modeling scenarios on various parts of time-of-flight mass spectrometers, ion guides, and ion optics. Designed, developed, and coded software packages including: (1) "MoleculeMaker™" for generating theoretical isotope profiles and electrospray spectra from elemental formulas or amino acid sequences, (2) "Balancer" for calculating sample concentrations and dilution sequences, and (3) numerous other small programs and spread sheets. Project manager, which included supervising internal and external computer programmers, for a commercialized software package, "MacroMass™," used for deconvoluting multiply-charged electrospray mass spectra.

University of California, San Diego, California; Graduate Research Assistant; 1984 - 1991.

Ph.D. dissertation on metal cluster beams and magnetic thin films. Designed and constructed the following instruments: (1) Ultra High Vacuum (UHV) system, (2) 2200°C nozzle for metal beam film growth, and (3) UHV M-H Magnetometer. Analyzed thin films by Auger Electron Spectroscopy (AES), Low Energy Electron Diffraction (LEED), Temperature Program Mass Desorption (TPD), M-H Magnetometry, Electron Spectroscopy for Chemical Analysis (ESCA), and Transmission Electron Microscopy (TEM). Interfaced an Auger Electron Spectrometer, a M-H Magnetometer, and a Mass Spectrometer for temperature program mass desorption, to an IBM PC controller.

Lycoming College, Williamsport, Pennsylvania; Computer Assistant Manager; 1981 - 1984.

Responsibilities included tutoring, programming, systems operations, and supervision of assistants.

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EDUCATION:

Ph.D., University of California, San Diego, 1991. Engineering Physics.
M.S., University of California, San Diego, 1986. Engineering Physics, (GPA) 3.54.
B.A., Lycoming College, Pennsylvania, 1984. Computer Science (GPA) 4.00, Physics (GPA) 3.88, Magna Cum Laude (GPA) 3.69.

FELLOWSHIPS AND HONORS:

Dissertation Research Fellowship, 1991.
University of California Regents Fellowship, 1984-1985.
Charles J. Kocian Computer Science Award, 1984.
Ferree Mathematical Sciences Award, 1984.
Phi Kappa Phi National Honor Society, 1984.
Lycoming Scholar, 1980-1984.
Dean's List, 1980-1981, 1983-1984.
Sigma Pi Sigma National Physics Honor Society, 1983.
Blue Key Superior Academic Freshman Award, 1981.

RELATED ACTIVITIES:

Society of Physics Students, Lycoming College, 1980-1984.
Student Advisory Committee for Mathematical Sciences, Lycoming College, 1983-1984.
Student Judiciary Appointment Board, Lycoming College, 1983-1984.
ACM Allegheny Region Programming Contest, 1982 and 1983.
SHARE program, observed engineering and computer departments at AVCO Lycoming, 1983.

PROFESSIONAL SOCIETIES:

American Society for Mass Spectrometry, American Chemical Society, American Vacuum Society, Materials Research Society, Phi Kappa Phi.

SPECIAL SKILLS:

Proteomics, Electrospray, Atmospheric Pressure Chemical Ionization (APCI), Time-of-Flight Mass Spectrometry (TOF-MS), Mass Spectrometry (MS), Molecular Beams, CAD, SPR BIACORE, Ion Mobility, AFFF/MALS, Visual Basic, computer programming.

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SEMINARS:

Invited guest speaker at WATERS Japanese Executive Technology Forum 2009
Connecticut Separation Science Council (CSSC) Monthly Meetings (2001-present)
CT Mass Spectrometry Monthly Discussion Group (1991-2001)
Fenn 90 Symposium 2007
Discovery Proteomics, New Mass Spectrometry, Biomarker, Quantitation and PTM Analysis, Applied Biosystems, May 4, 2004
Invited Speaker for Amersham Biosciences European Proteomics Tour, (Summer 2001)

SHORT COURSES:

Practical Mass Spectrometric Characterization of Protein Therapeutics, (ASMS 2012)
Proteins Structural Analysis By Mass Spectrometry, (ASMS 2011)
MALDI Imaging Mass Spectrometry: Basic Tools and Techniques, (ASMS 2010)
Metabolomics (ASMS 2009)
Wyatt Light Scattering University (2007)
Quantitative MS, (ASMS 2007)
Proteins and Peptides by Mass Spectrometry, (ASMS 2005)
Biacore University SPR Basics Customer Training (April 2005)
Mass Spectrometry in Drug Discovery (ASMS 2004)
Applied BioSystems Voyager De-Pro Customer Training, (December 2002)
Bruker Esquire Ion Trap Customer Training, (April 2002)
Agilent 1100 HPLC ChemStation Customer Training, (Spring 2002)
Well Characterized Biologicals, (IBC's 5th International Conference 2001)
Characterization of Proteins and Peptides by Mass Spectrometry, (ASMS 2001)
Advanced SIMION 7.0 (ASMS 2000).
Fundamentals and Applications of MALDI-TOF-MS (ASMS 1999).
High Performance Liquid Chromatography, HPLC, and LC/MS (EAS 1998)
Quadrupole Ion Trap and Tandem Mass Spectrometry (ASMS 1998).
PHI Surface Analysis and Instrumentation Course on ESCA, (1987).

CONFERENCES ATTENDED:

American Society of Mass Spectrometry Conference, (1992-2012)
Well Characterized Biologicals, (IBC's Conference 2001 and 2009)
Next Generation Biologics, (IBC's Conference 2009)
Csaba Horvath Memorial Symposium (2005, 2007, 2009)
18th Annual International Light Scattering Colloquium(2007)
DiPIA 2007 Developments in Protein Interaction Analysis Biacore 2007
Pittsburgh Conference, (1993-2001)

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ALEXION TRAINING:

GMP Training (2001-2015)
Presentation Skills (30Jul08)
Intercultural Training for Filing in Japan (29Jul08)
21 CFR Part 11 (General Awareness Training) (14Jun07)
Tips, Techniques & Shortcuts for Organizing & Maintaining Files & Records (Skillpath) (17Oct06)
Change Control Training (SOP-QA-0038), (11Jul06)
SAP Training, (07Jul06)
Performance Management Process Appraisal Writing & Delivery Training, (30Jun06)
SharePoint Portal Server 2003 Training, (16Jun06)
On-Site Regulatory Inspections (SOP-RA-1003.00), (April 3, 2006)
Change Control Program (SOP-QA-0038.03), (10Mar06)
cGMP Briefing Presentation for Pharmaceutical Stakeholders, (02Mar06)
CP-0001.00, CP-0002.00, CP-0003.00 (16Nov05)
WPD-TS-001-00, WPD-TS-002-00, WPD-TS-003.01 (16Nov05)
Coaching and Teambuilding Skills for Managers and Supervisors, SkillPath (June 15, 2005)
Goal Setting for Groups, SkillPath (May 18, 2005)
Excelling as a First-Time Manager, SkillPath (January 21, 2005)
VBA for MS Excel, (November 10 and 19, December 15, 2004)
Fundamentals of Successful Project Management, SkillPath, (November 3, 2004)
SOP-QA-0001.05, 0002.04, 0003.05, 0017.05 0022.05 Doc Control (04Jun04)
Excel Microsoft XP- Pivot Tables, (April 2004)
Excel Microsoft XP- Formulas, (March 2004)
RDM-0033.00, Determination of Eculizumab Purity by SEC-HPLC for In Process Testing (09Feb04)
SOP-CAL-0029.00 Standardization of the AND Electronic Balance, Model #FD-6000 (09Feb04)
RDM-0012.00 Quantification of Protein Concentration in Pexelizumab Samples by UV-Vis Spectrophotometry (03Feb04)

Sexual Harassment Prevention Training, (2003)
SOP-QA-0055.00 Labaratory Notebook Requisition, Issuance, and Tracking (22Dec03)
Laboratory Notebook Documentation, Jeff Gassman, (09Dec03)
PowerPoint Microsoft, Key-Logic, Dynamic Slide Enhancements, (28Oct03)
SOP-QCG-0025.03 Quality Control Sample Submission and Handling (20Oct03)
SOP-QA-0017.04 Document Control System, (04Aug03)
Intro to Good Scientific Practices (GSP), (23May03)
SOP-QA-0043.01, (21Jan03)

SOP-QA-0001.03, 0002.02, 0003.02, 0004.02, 0017.03, and 0022.03, (10Nov02)
Document Control System, Controlled Copy of SOP, and SOP Training (11Oct02)
21 CFR Compliance Training Session II; cGMP, GLP, GCP, and QA, (24Jun02)
Pipettes Training, Alpha-Omega, (29May02)
Basic Good Laboratory Practices (21 CFR Part 58), (28May02)

cGMP's and Compliance Document Control System, (17Sep01)
Annual OSHA Training/Safety Training, (Mar 2006, 2005, 2004, 2003, 2002, 2001)

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PUBLICATIONS AND PRESENTATIONS:

"Kinetic analysis of a monoclonal therapeutic antibody and its single-chain homolog by surface plasmon resonance", Rekha Patel *, Bruce A. Andrien Jr., Anal. Biochem. 396 (2010) 59–68.

"Kinetic Analysis of Anti-C5 Subclasses to Gamma Receptors by Surface Plasmon Resonance", Rekha Patel, Bruce Andrien, Krista Johnson, Jeff Hunter, Pat Bento, Michelle Giannoni and Paul Tamburini, Developments in Protein Interaction Conference 2009

"The Role of LC/MS to Support Biotechnological/Biological Product Regulatory Submissions", Invited guest speaker at WATERS Japanese Executive Technology Forum 2009

"AFFF/MALS and Ion Mobility for Analysis of Antibodies and Non-Covalent Complexes", Bruce Andrien, Csaba Horvath Symposium 2009

"Integration of Waters BiopharmaLynx Application Manager into a Monoclonal Antibody Characterization Strategy", Adam Lucka, Rekha Patel, Christine Nowak, and Bruce Andrien, WATERS users meeting at the American Society of Mass Spectrometry Conference 2009

"Ion Mobility Spectrometry for Determining Binding, Bridging and the Formation of Non-Covalent Complexes", Bruce Andrien, Rekha Patel, Christine Nowak, and Adam Lucka, American Society of Mass Spectrometry Conference 2009

"Characterizing Five Deamidation Products from a Single Asparagine Residue in Recombinant mAb's Through LC-MS Peptide Mapping", Adam Lucka, Christine Nowak, Rekha Patel, and Bruce Andrien, American Society of Mass Spectrometry Conference 2009

"Analysis of Antibodies, Aggregates and Non-Covalent Complexes by IMS, SEC/MALS/IMS and AFFF/MALS/IMS", Bruce Andrien, Christine Nowak, and Adam Lucka, Invited Guest Speaker at TSI User Meeting at ASMS Conference, (2008).

"Coupling AFFF/MALS or SEC/MALS to Ion Mobility Spectrometry for Analysis of Antibodies, Fragments, Aggregates and Non-Covalent Complexes", Bruce Andrien, Christine Nowak, and Adam Lucka, 56th ASMS Conference, (2008).

"Characterizing Glycosylation Patterns in Recombinant IgG2/4 Monoclonal Antibodies by Combining Fluorescence HPLC, MALDI-ToF-MS, and LC-MS and MS/MS Data", Adam Lucka, Christine Nowak, Rekha Patel, and Bruce Andrien, 56th ASMS Conference, (2008).

"An Investigation of Protein Therapeutics Including Antibodies and Antibody Conjugates with Ion Mobility Spectrometry", Bruce A. Andrien, Rekha Patel, Lynellen Willard, and Rachael Alford, 55th ASMS Conference, (2007).

"Improving Monoclonal Antibody and SCFv Protein Characterization by Obtaining Complementary MALDI-TOF-MS Spectra from a Single Tryptic Digest", Adam Lucka, Rekha Patel, and Bruce Andrien, 55th ASMS Conference, (2007).

"Kinetic Analysis of a Monoclonal Antibody (eculizumab) and its Single Chain Homolog (pexelizumab) by Surface Plasmon Resonance", Rekha Patel and Bruce Andrien, DiPIA Conference 2007.

"Disulfide Bond Confirmation of a Humanized Anti-C5 Complement Monoclonal Antibody and Its Single Chain Homolog by Peptide Mapping", Bruce A. Andrien, Adam W. Lucka, and Rekha Patel, 54th ASMS Conference, (2006).

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"Characterization of a Monoclonal Antibody and its Single Chain Therapeutic", Rekha Patel, Adam Lucka, and Bruce Andrien, Invited Guest Speaker at Agilent World Tour 2006, Cambridge, MA, (16May06).

"Workflow for the Characterization of Proteins Therapeutics", Bruce A. Andrien, Jr., Adam Lucka, Rekha Patel, Invited Guest Speaker at Agilent Lunch and Learn during 53th ASMS Conference, (2005).

"A Bioinformatics Strategy for the Characterization of Proteins versus Protein Identification", Bruce A. Andrien, Jr., Adam Lucka, Rekha Patel, 53th ASMS Conference, (2005).

"Improving Protein Coverage in nanoLC-MS Peptide Mapping by using a Sub-Ambient Enrichment Column to Bind Hydrophilic Peptides", Adam Lucka, Rekha Patel, Bruce A. Andrien, Jr., 53th ASMS Conference, (2005).

"Protein Identification after Two-Dimensional Gel Electrophoresis by MALDI ToF MS and LC ESI ToF MS", Staffan Renlund; Ylva Laurin; Maria Liminga; Henrik Wadensten; Bruce A. Andrien, Jr.; Marketa Berkova; Erol E. Gulcicek, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Confirmation Analysis of Protein Purification using Electrospray Time of Flight Mass Spectrometry", Henrik Wadensten; Ninni Salomonsson; Christel Ellström; Staffan Lindqvist; Staffan Renlund; Bruce A. Andrien, Jr.; Marketa Berkova; Erol E. Gulcicek, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Evaluation of Rugged Microelectrospray Iinterfaces for Identification of Peptides and Proteins Wiith APII-TOF-MS", Erol E. Gulcicek; Bruce A. Andrien, Jr; Marketa Berkova; Craig M. Whitehouse; Staffan Renlund, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Investigating the Trap-Pulse Mode of Operation to Improve the Sensitivity of Electrospray Time-Of-Flight Mass Spectrometer", Marketa Berkova; Craig Whitehouse; Erol Gulcicek; Bruce Andrien, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Identification of Post-Translational Modifications by Fast LC-MS and In-Source CID Using a New API TOF MS", Staffan Renlund, Henrik Wadensten, Lars Rönnstrand, Rannar Sillard, Bruce A . Andrien, Jr., Marketa Berkova, and Erol E. Gulcicek, Montreux (2000).

"Optimization of Rapid State Switching API TOF Operation for Fast LC-MS", Erol E. Gulcicek, Bruce A. Andrien, Marketa Berkova, James G. Boyle, Shida Shen, and Craig M. Whitehouse, and Staffan Renlund, Montreux (2000).

"Peptide and Protein Analysis and the Identification of Post-Translational Modifications Using Fast LC-MS with Rapid State Switching", Craig M. Whitehouse, Bruce A. Andrien, Jr., Staffan Renlund, Erol Gulcicek, Marketa Berkova, Lars Ronnstrand, and Rannar Sillard, 15th IMSC Barcelona (2000).

"Identification of Proteins and Post Translational Modifications Using New MALDI ToF and ESI ToF Mass Spectrometer Systems", Staffan Renlund, Jonas Åström, Henrik Wadensten, Rannar Sillard, Lars Rönnstrand, Bruce A. Andrien, Jr., Marketa Berkova, and Erol E. Gulcicek, 4th Int Seminar on Biomolecule Identification and Function (2000).

"Fast LC-MS for Peptide Mass Mapping, Protein Analysis and Identification of Post-Translational Modification Using API TOF", S. Renlund, H. Wadensten, J. Astrom, J. Ericsson, S. Ekman, U. Engstrom, L. Ronnstrand, B. A. Andrien, E. E. Gulcicek, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

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"Analysis of Proteins from 2D-Gels by MALDI-TOF MS and LC-ESI-TOF MS", M. Raida, G. Reil, A. Gorg, J. Astrom, H. Wadensten, S. Renlund, and B. A. Andrien, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

"Combining Autoinjector and Nebulizer Hardware for Enhanced Automation in FIA-MS,", B. A. Andrien, J. G. Boyle, and M. Berkova, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

"Multipole Storage Assisted Dissociation for Enhancing Sequence and Structural Studies in LC-TOF-MS", C. M. Whitehouse, B. A. Andrien, and M. Berkova, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

"Evaluation of a New LC TOF-MS with Wide Dynamic Range for Full Chromatographic Compatibility", B. A. Andrien, E. E. Gulcicek, S. Shen, C. M. Whitehouse, and J. G. Boyle, Pittsburgh Conference Proceedings, (2000).

"High Performance at High Throughput Lessons from LC-API-TOF-MS", E. G. Gulcicek, B. A. Andrien S. Shen, C. M. Whitehouse, and J. G. Boyle, Pittsburgh Conference Proceedings, (2000).

"Increasing the Duty Cycle for Time-of-Flight by Trap-Pulse Mode", B. A. Andrien, C. M. Whitehouse, E. E. Gulcicek, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Rapid API TOF State Switching with Fast LC-MS", C. M. Whitehouse, E. E. Gulcicek, B. A. Andrien, S. Shen, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Obtaining High Mass Accuracy on LC-TOF-MS", E. E. Gulcicek, C. M. Whitehouse, B. A. Andrien, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Dual Parallel Probes for Electrospray Sources", S. Shen, B. A. Andrien, M. Sansone, and C. M. Whitehouse, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Multiple Inlet Probes for Electrospray and APCI Sources", B. A. Andrien, M.A. Sansone, and C. M. Whitehouse, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"Minimizing Chemical Noise through Rational Design of a "Universal" API Source", S. Shen, B. Andrien Jr., M. Sansone, and C. M. Whitehouse, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"A Two Dimensional Ion Trap API TOF Mass Spectrometer", C. M. Whitehouse, E. E. Gulcicek, J.F. Banks, B. Andrien, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"Performance of High Throughput API TOF MS Detector", E. E. Gulcicek, B. A. Andrien, J.F. Banks, J.G. Boyle, and C. M. Whitehouse, 10th Sanibel Conference on Mass Spectrometry, Sanibel, FL, (1998).

"Atmospheric Pressure Chemical Ionization and Electron Capture Negative Chemical Ionization Mass Spectrometry in Studying ß-Carotene Conversion to Retinol in Humans", Guangwen Tang, Bruce A. Andrien, Gregory G. Dolnikowski, and R. M. Russell, Methods in Enzymology, **282**, 140-154, (1998).

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"A Disposable μ-Tip Probe for Low Flow Electrospray", B. A. Andrien, D. M. Sauro, M.A. Sansone, G.P Whitehouse, C. M. Whitehouse, and A.G. Burt, 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

"Performance of a Hexapole Ion Guide on HP5988", B. A. Andrien, M.A. Sansone, B.L. Mancini, C. M. Whitehouse, A.G. Burt, Guangwen Tang, and Gregory G. Dolnikowski 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

"Emerging Biomedical High Throughput Sample Inlet Systems Utilizing an API TOF MS Detector", E. E. Gulcicek, B. A. Andrien, J.F. Banks, J.G. Boyle, and C. M. Whitehouse, 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

"Detecting the *in vivo* Conversion of b-Carotene-d_8 to Retinol-d_4 by APCI-MS and GC/ECNCI-MS", Guangwen Tang, Bruce A. Andrien, and Gregory G. Dolnikowski, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).
"Atmospheric Pressure Ionization Flow Injection Analysis by Orthogonal Time-of-Flight Mass Spectrometry", B.A. Andrien, T. Dresch, P.J. Haren, and C. M. Whitehouse, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).

"On-Line Detection of Fast Chromatographic Events by Electrospray Ionization Time-of-Flight Mass Spectrometry", Erol E. Gulcicek, B. Andrien, J.F. Banks, J.G.Boyle, T.Dresch, P.Haren,and C.Whitehouse, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).

"Micro and Nano Flow Atmospheric Pressure Chemical Ionization", B. A. Andrien and C. M. Whitehouse, Pittsburgh Conference Proceedings, (1996).

"API Time of Flight Mass Spectrometry as a Chromatography Detector", B. Andrien, J. F. Banks, T.Dresch, E.Gulcicek, P.Haren, J.Quinn, and C.Whitehouse, Pittsburgh Conference Proceedings, (1996).

"Electrospray and APCI Interface for Mass Spectrometry", B. A. Andrien and J. G. Boyle, *Spectroscopy*, **10**, 2, 42-44, (1995).

"Improvements to Electrospray and APCI Ion Sources for CE/MS and LC/MS Applications with TOF, Quadrupoles, FTMS, Ion Traps and Magnetic Sector Spectrometers" C. M. Whitehouse, J. F. Banks, E.Gulcicek, T.Dresch, B. Andrien, and S. Shen, 37th Rocky Mountain Conference on Analytical Chemistry, Denver, CO, (1995).

"Atmospheric Pressure Chemical Ionization by Corona Discharge", B. A. Andrien, T. Dresch, P.J. Haren, C. M. Whitehouse, and S. Shen, 43th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Atlanta GA (1995).

"A Mass Analyzer for High Speed Liquid Chromatography", B. A. Andrien, J. F. Banks, J. G. Bolye, T. Dresch, P.J. Haren, and C. M. Whitehouse, 43th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Atlanta GA (1995).

"Performance of an Atmospheric Pressure Corona Discharge Ion Source with Time of Flight Mass Spectrometry", B. A. Andrien, T. Dresch, P.J. Haren, and C. M. Whitehouse, Pittsburgh Conference Proceedings, (1995).

"Atmospheric Pressure Chemical Ionization by Corona Discharge with Pneumatic and Ultrasonic Nebulization", B. A. Andrien, J. P. Quinn, and C. M. Whitehouse, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

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"Theoretical and Practical Considerations for Determining Resolution and Conducting Mass Calibration with Multiply Charged Ions", B. A. Andrien, P.J. Haren, and C. M. Whitehouse, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

"Improved Performance of a New Electrospray and APCI source Interfaced to a ZAB or 7070 Series Magnetic Sector Mass Spectrometer", S. Shen, C. M. Whitehouse, B. A. Andrien, and Walter McMurrary, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

"Performance of an Atmospheric Pressure Corona Discharge Ion Source with Ultrasonic Nebulization", B. A. Andrien, J. P. Quinn, and C. M. Whitehouse, Pittsburgh Conference Proceedings, Chicago (1994).
"Performance of an Atmospheric Pressure Ion Source with Corona Discharge and Ultrasonic Nebulization", B. A. Andrien, C. M. Whitehouse, and J. P. Quinn, 41th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, San Francisco (1993).

"Deconvolution of Electrospray Generated Multiply Charged Ion Spectra Using a Three Dimensional Technique", B. A. Andrien, M. Labowski, and C. M. Whitehouse, The Pittsburgh Conference Proceedings (1993).

"A Comparison of Magnetic Properties of Thin Films of Fe/GaAs(100) and Ni/Mica", B. Andrien and D.R. Miller, Materials Research Society Symposia Proceedings (submitted Dec. 1992). Fall Meeting, Boston, MA, Dec. 1992.

"Deconvolution of Electrospray Generated Multiply Charged Spectra of Mixtures Using a Three Dimensional Technique", B. A. Andrien, M. Labowski, and C. M. Whitehouse, 40th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (1992). Washington, DC (1992).

"Thin Film Growth and Magnetic Properties for Fe/GaAs(100)", B. Andrien and D.R. Miller, Materials Research Society Symposia Proceedings, **202**, 193 (1991). Fall Meeting, Boston, MA, Dec. 1990.

"Magnetization Loop Tracer for Superconductors", B. Andrien and J. Haugdahl, Review of Scientific Instruments **61(8)**, 2260 (1990).

"Chemisorption on Ferromagnetic Metal Films", B. Andrien and D.R. Miller, Materials Research Society, Spring Meeting, San Diego, CA, Apr. 1989.

"Thin Ferromagnetic Metal Cluster Films", B. Andrien and D.R. Miller, American Vacuum Society Vacuum Metallurgy Division, 15th International Conference on Metallurgical Coatings, San Diego, CA, Apr. 1988.

"Magnetic Properties of Thin Metal Cluster Films", B. Andrien, J. Haugdahl, and D.R. Miller, Journal of Vacuum Science and Technology A **6(3)**, 1865 (May 1988). American Vacuum Society, 34th National Symposium, Anaheim, CA, Nov. 1987.

"A Spectroscopic Investigation of Van Der Waals Complexes", B. Andrien, 38th Eastern Colleges Science Conference, Providence College, RI, Apr. 1984.

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AWARDED PATENTS:

Title: "Nucleic Acid Encoding Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,206,251 B2
Date of Patent: Dec. 8, 2015

Title: "Methods of Treating C5 Mediated Complement-Associated Conditions with Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,107,861 B1
Date of Patent: Aug. 18, 2015

Title: "Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,079,949 B1; WO 2015/134894 A1; New Zealand 631007; European 15 710 396.1
Date of Patent: Jul. 14, 2015; 06Mar. 2015; 02Feb2016; 06.03.2015

Title: "Mass Spectrometry with Multipole Ion Guides"
Inventors: Craig M. Whitehouse, Bruce A. Andrien, Jr.; and Gulcicek; Erol E.
Patent No.: 6,753,523; 6,987,264; and 7,189,967
Date of Patent: June 22, 2004

Title: "Atmospheric Pressure Ion Source"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr., Michael A. Sansone, Clement Catalano
Patent No.: 6,593,568 B1
Date of Patent: July 15, 2003

Title: "Direct Flow Injection Analysis Nebulization Electrospray and APCI Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Fred Banks, James Boyle
Patent No.: 6,458,597 B1; 6,858,437 B2; and 7,364,913
Date of Patent: October 1, 2002; and Feb 22, 2005

Title: "Curved Introduction for Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Michael A. Sansone, Allan G. Burt, Craig M. Whitehouse
Patent No.: 6,326,616 B1 and 6,573,494 B1
Date of Patent: December 4, 2001 and June 3, 2003

Title: "Multiple Sample Introduction Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Shida Shen, Michael A. Sansone,
Patent No.: 6,207,954 B1 and 6,541,768 B2
Date of Patent: Mar 27, 2001 and April 1, 2003

Title: "Disposable Microtip Probe for Low Flow Electrospray"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Michael A. Sansone, Denise M. Sauro, Glenn P. Whitehouse
Patent No.: 6,127,680 and 6,337,480 B1
Date of Patent: October 3, 2000 and January 8, 2002

Title: "Time-Of-Flight Mass Spectrometry from Surfaces"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr.
Patent No.: 6,040,575 and 6,204,500 B1 and 6,600,155 B1
Date of Patent: March 21, 2000 and Jul 29 ,2003

Title: "Multipole Ion Guide Trap Time-of-Flight Mass Spectrometry with MS/MS^n Analysis Capability"
Inventors: Craig M. Whitehouse, Thomas Dresch, and Bruce A. Andrien Jr.
Patent No.: 6,011,259
Date of Patent: January 4, 2000

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PRINCIPLE INVESTIGATOR for the FOLLOWING GRANTS:

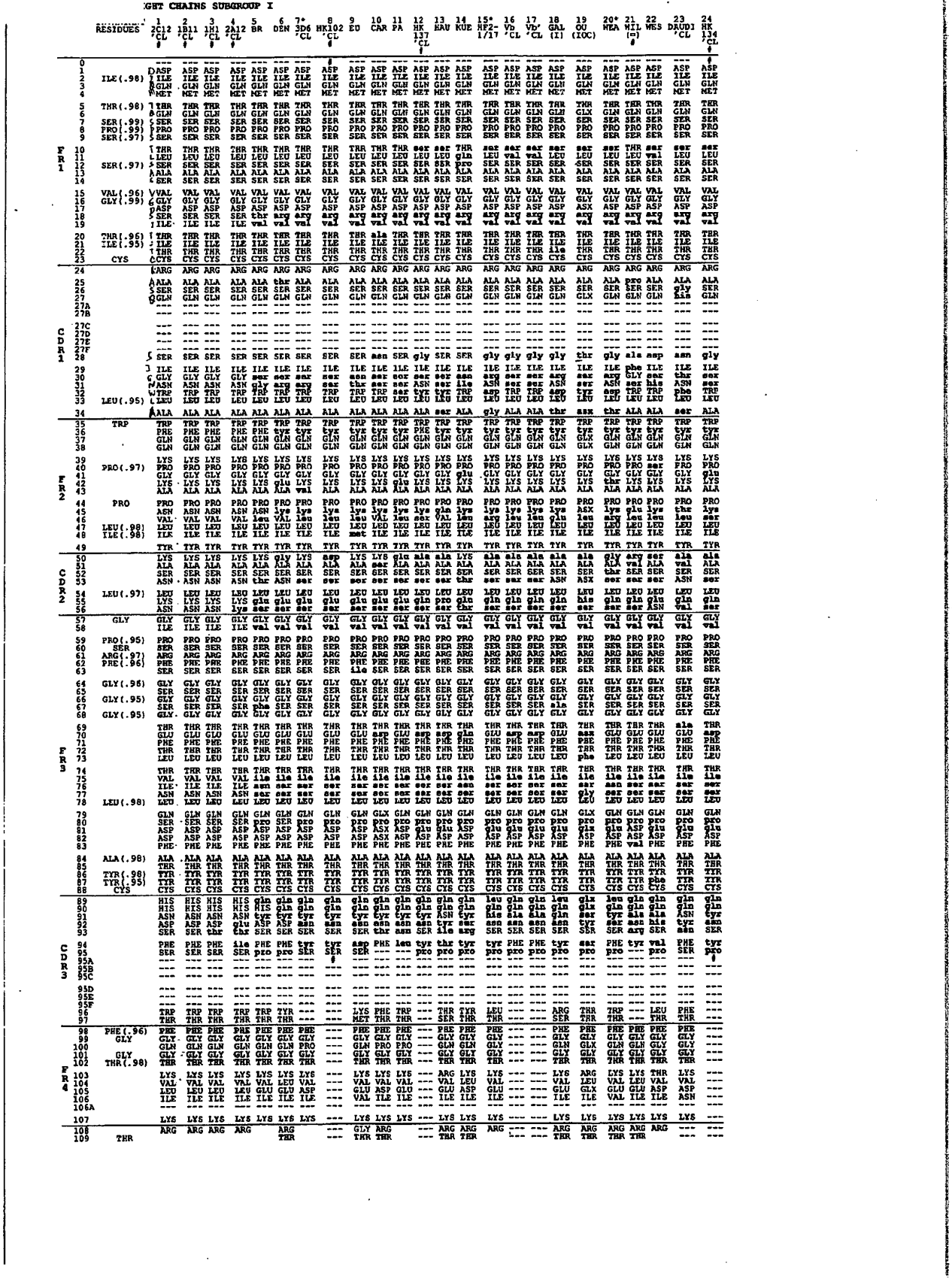
Title: "Microflow APCI Method for High Throughput LC-MS"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 GMS6604-01; Awarded: $90,000.00
Support Period: 9/1/97-3/1/98
Description: To Develop a microflow APCI interface for mass spectrometry to improve the limits of detection for trace non-polar species in complex mixtures, while at the same time making on-line coupling to microbore chromatographic methods easier, more reproducible and robust.

Title: "MSD Mass Detector with Atmospheric Pressure Inlet"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 RR12058-01; Awarded: $90,000.00
Support Period: 9/1/97-9/1/98
Description: Develop a platform which will provide a high productivity, low cost entry point for both LC and CE users, as well as for GC-MS labs needing the ability to handle non-volatile samples without requiring time consuming derivitization procedures.

Title: "Mass Spectrometer Detector for Liquid Chromatography"
Funding NIH Phase I and II (national Center for Research Resources)
ID Grant #: 1 R 43 RR08538-01, 2 R 44 RR092252-02; Awarded: $50,000, $500,000
Support Period: 3/1/94-8/31/94, 4/1/95-3/31/97
Description: Develop a pre-production prototype API-TOF-MS bench top system for non mass spectrometrist. It should connect to any LC or CE system and ionizes the sample by APCI and ESI to analyze polar and non-polar samples.

Title: "Deconvolution of Mass Spectra for Multiply Charged Ions"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 RR08538-01; Awarded: $50,000.00
Support Period: 9/30/92-3/29/93
Description: To Develop a 3D software package that will take full advantage of the coherent pattern for determining the mass of higher molecular weight compounds from a series of multiply charged peaks produced by any multiply charged ionization process.

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GHT 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 PA
12 HK 137 'CL #
13 HAU
14 KUE
15* HEZ- 1/17
16 V9 'CL
17 V8' 'CL
18 GAL (I)
19 OU (IOC)
20* WEA
21 WIL (=) #
22 WES
23 DAUDI 'CL
24 HK 134 'CL #
F R 1
C D R 2
C D R 3
FR 1
FR 2
FR 3
FR 4

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		25 Vd 'CL	26 WALKER 'CL	27 HK101 'CL #	28 Va' 'CL	29 VKI-Chel 'CL	30 AMYLOID BAN #	31 Ve 'CL	32 V13 'CL	33 DEE	34 WIL (-) #	35 AND #	36 V108 'CL #	37 VKI-ZI 'CL	38 REI	39 AU	40 ROY	41 BI	42* HOVH CAMP 'CL	43 AG #	44 SCH	45 MEV	46 KA	47* LAY	48 BEL	49 NI
	0	---	---	#	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	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
	3	GLN	GLN	GLN	GLN	GLN	GLN	asn	trp	GLX	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	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
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	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
	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
F	10	phe	ser	ser	ser	ser	ser	ser	leu	ser	THR	ser	ser	ser	ser	ser	ser	pro	ser	ser	ser	ser	THR	ser	ser
R	11	LEU	LEU	LEU	LEU	LEU	LEU	phe	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
1	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	val	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	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
	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	ASP	ASP	ASP	ASP	ASP	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	SER	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val
	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
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	phe	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu
	22	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
	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
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	met	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	SER	SER	arg	SER	SER	SER	SER	SER	gly	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	GLX	GLN
	27A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ser
	27B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	val
C	27C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	leu
D	27D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	glu
R	27E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1	27F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	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	tyr	ser	ser	asn	ser	GLY	ser	ser	ile	ser	ser	asn	asp	asn	asp	val	leu	asn	ser	asn
	31	ser	ASN	ser	ser	ASN	ASN	ser	ser	lys	tyr	gly	ASN	ASN	lys	asp	ile	ASN	lys	his	lys	asp	ser	ala	lys	thr
	32	tyr	TYR	TYR	ala	asp	tyr	TYR	tyr	tyr	TYR	ser	gly	ser	tyr	tyr	phe	ser	tyr	tyr	tyr	tyr	tyr	tyr		phe
	33	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU
	34	ALA	asn	ALA	ALA	asn	ALA	ALA	ALA	asn	ALA	asn	ser	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	tyr	tyr	tyr	tyr	tyr	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	asp	GLN	GLN	GLN	GLN		GLN
	38	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN
	39	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	thr	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
	41	GLY	GLY	glu	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	$	GLY	GLY	GLY	GLY	GLY	lys
F	42	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	gln	LYS	LYS	LYS	LYS	LYS	LYS	leu	LYS	LYS	LYS	LYS	LYS	LYS
R	43	ALA	ALA	ALA	ALA	thr	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	thr	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	pro	ALA
2	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	lys	lys	lys	lys	glu	lys	ASN	ser	thr	lys	lys	lys	lys	lys	lys	arg	lys	lys	lys	glu	lys	
	46	leu	leu	ser	leu	leu	leu	leu	leu	VAL	leu	leu	leu	leu	leu	ile	leu	phe	leu	leu	leu	leu	leu	leu	leu	
	47	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	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	TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR
	50	ala	ala	ala	asp	ala	asp	ala	ala	ala	gln	gly	ala	ala	glu	asp	asp	asp	asn	asp	gly	asp	ala	gly	asp	asp
	51	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	thr	ALA	ALA	ALA	ALA	ALA
C	52	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	asn	SER	SER	SER	SER	SER	SER	SER
D	53	thr	ser	ser	ser	ser	thr	thr	thr	ser	ile	ser	ser	ser	ASN	ASN	lys	ASN	ASN	ASN	thr	ASN	ser	thr	thr	ASN
R	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
2	55	gln	gln	gln	glu	gln	gln	gln	gln	LYS	asp	arg	gln	gln	gln	glu	glu	glu	gln	glu	glu	gln	glu	gln	LYS	glu
	56	ser	ser	ser	ser	ser	ser	ser	ser	ser	ala	ser	ser	ser	ala	ser	ala	ile	thr	thr	thr	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	val	val	val	val	ILE	val	val	val	val	val	val	val	ILE	val	val	val	val	val	val	val	val	val	val	val	val
	59	PRO	thr	PRO	PRO	SER	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	SER	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	60	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
	61	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
	62	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
	63	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	arg	SER	SER	SER	SER	SER	SER	SER	SER
	64	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
	65	SER	SER	SER	SER	SER	SER	SER	SER	SER	asn	SER	SER	SER	SER	gly	thr	SER	SER	SER	gly	gln	SER	SER	SER	SER
	66	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	ile	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY
	67	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	phe	SER	SER	SER	SER	SER	SER
	68	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	glu	GLY
	69	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	ala	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	70	GLU	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	bis	asp	asp	asp	asp	asp	asx	asp	bis	asp	
	71	PHE	PHE	PHE	PHE	tyr	PHE	PHE	PHE	PHE	PHE	PHE	PHE	tyr	tyr	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	
F	72	THR	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR
R	73	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	phe	phe	phe	LEU	LEU	LEU	LEU	phe	LEU	LEU	phe
3	74	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR
	75	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile
	76	ser	ser	ser	ser	arg	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser
	77	ser	ser	ser	ser	ser	ser	tyr	gly	ser	tyr	ser	ser	ser	ser	ser	ser	gly	ser	gly	ser	ser	ser	ser	gly	gly
	78	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU	LEU	LEU
	79	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	leu	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLX
	80	pro	pro	pro	pro	pro	pro	SER	SER	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro
	81	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glx	glu	ala	glx	
	82	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	asn	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP
	83	PHE	ser	PHE	PHE	PHE	PHE	PHE	PHE	PHE	ser	PHE	val	PHE	ile	ile	ile	ile	ile	PHE	PHE	PHE	ile	PHE	PHE	PHE
	84	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	gly	ALA	ALA	ALA	ALA	ALA
	85	THR	THR	THR	THR	THR	THR	THR	THR	THR	arg	ala	THR	ala	THR	THR	THR	THR	THR	THR	asn	THR	THR	THR	val	
	86	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	87	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	88	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	89	gln	gln	gln	gln	gln	gln	gln	gln	glx	gln	gln	leu	gln	gln	gln	gln	gln	leu	gln	gln	gln	gln	gln	gln	gln
	90	gln	gln	gln	gln	gln	gln	gln	gln	glx	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	glx
	91	leu	ser	tyr	phe	ser	tyr	tyr	tyr	ser	tyr	ser	asp	ser	tyr	tyr	phe	tyr	his	tyr	tyr	ser	tyr	tyr	tyr	tyr
	92	asn	tyr	THR	asn	ASP	asn	tyr	tyr	asn	asn	tyr	ser	ASP	ASP	ASP	ASP	tyr	ile	ASP	ASP	tyr	leu	asn	ASX	ASP
	93	SER	SER	SER	SER	SER	SER	SER	SER	thr	arg	SER	thr	SER	SER	tyr	asn	asn	SER	thr	asn	thr	asp	asn	his	thr
C	94	tyr	thr	tyr	tyr	thr	tyr	tyr	PHE	thr	tyr	ser	thr	thr	leu	leu	len	leu	arg	leu	val	asn	leu	trp	PHE	leu
D	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
R	95A	---	---	#	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	GLU	---	---	---	---	---
3	95B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	96	---	ILE	---	---	---	TYR	---	---	TYR	---	TRP	---	---	TYR	TRP	LEU	TYR	ARG	ARG	ILE	VAL	ARG	PRO	LEU	SER
	97	---	THR	---	---	---	THR	---	---	THR	---	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	98	---	PHE	---	---	---	PHE	---	---	PHE	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	GLY	GLY	GLY
	100	---	GLN	---	---	---	GLN	---	---	PRO	GLN	GLN	---	---	GLN	GLN	GLY	GLN	GLN	GLN	GLN	GLY	GLN	GLN	GLY	VAL
	101	---	GLY	---	---	---	GLY	---	---	GLY	GLY	GLY	---	---	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	102	---	THR	---	---	---	THR	---	---	THR	THR	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	SER
F	103	---	ARG	---	---	---	LYS	---	---	LYS	LYS	LYS	---	---	LYS	LYS	LYS	LYS	LYS	LYS	ARG	THR	LYS	LYS	GLU	LYS
R	104	---	LEU	---	---	---	#	---	---	#	VAL	LEU	---	---	VAL	LEU	VAL	LEU	VAL	LEU	VAL	VAL	VAL	VAL	VAL	VAL
4	105	---	GLU	---	---	---	#	---	---	#	GLU	GLU	---	---	GLN	GLU	ASP	GLU	GLU	GLU	GLU	ASP	ASP	GLU	GLU	GLU
	106	---	ILE	---	---	---	ILE	---	---	MET	ILE	ILE	---	---	ILE	ILE	PHE	ILE	ILE	ILE	ASN	ILE	LEU	VAL	VAL	SER
	106A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	107	---	LYS	---	---	---	LYS	---	---	THR	LYS	ARG	---	---	THR	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	108	---	---	---	---	---	ARG	---	---	ARG	ARG		---	---	ARG	ARG	ARG	ARG	ARG	ARG	GLY	ARG	ARG	ARG		ARG
	109	---	---	---	---	---	---	---	---	THR	THR		---	---	THR	THR	THR	THR	---	IRR	THR	---	THR	---	---	THR

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		50 Rz	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 48	63 HF3-16/6	64 HF2-1/13B	65 HF2* 18/2	66 GR'	67 GL 'GL #	68 HOM	69 KER	70 ESM IGG	71 ESH IGH	72* DAV	73* PIN	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	his	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN
	4	MET	leu	MET	MET	leu	MET	MET	MET	MET	MET	leu	MET	MET	MET	MET	MET	MET		MET	MET	MET	MET	MET	MEI	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	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	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	
R	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	
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	ALA	val
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	tyr	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	val	val	SER	SER	pha
	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	ASP	ASX	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP
	18	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary	ary		ary	ary	ary	ary	ary	ary	ary	ary	
	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	ILE	val	phe	ILE
	22	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	ary	THR	THR
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS		CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	gln	ARG	ARG	gln	gln	gln	gln	ARG	gln	ARG	glx	ARG		ARG	ARG	ARG	ARG		gln	gln	ARG	ARG	asp	asp	ARG	glx	glx
	25	ALA	ser	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	asn	SER	SER	SER	SER	asn	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER		arg	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	---	GLN	GLN	GLN	GLX	GLX		GLN	GLN	GLN	arg		hiS	GLN	GLX	GLX	GLN	GLN	GLX	GLX	GLX
	27A	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	27B	---	leu	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
C	27C	---	val	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
D	27D	---	hiS	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
R	27E	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
1	27F	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	28	val	asp	SER	SER	asp	val	---	SER	asp	SER	asx	asx		gly	gly	gly	SER		asp	asp	SER	SER	asx	asx	SER	asn	asx
	29	ILE	gly	val	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	30	ser	asn	asn	asn	asn	ser	arg	ser	arg	ser	ser	asx		arg	arg	arg	ser		ser	lys	ser	ser	asx	asx	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	pha	ser	tyr	ser	tyr	pha	tyr	tyr		asp	asp	asp	TRP		tyr		tyr	tyr	TRP	TRP	tyr	tyr	tyr
	33	LEU	LEU	LEU	ala	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU		LEU	LEU	LEU	LEU	ser	LEU	LEU
	34	asn	glu	ALA	asn	asn	asn	asn	ALA	asn	#	asx	ALA		gly	gly	gly	ALA		asn		asx	asx	ile	ile	asx	asx	asx
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP		TRP		TRP	TRP	TRP	TRP	TRP	TRP	TRP
	36	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr		tyr	tyr	tyr	tyr		PHE		tyr	tyr	tyr	tyr		tyr	tyr
	37	GLN	GLN	GLN	glu	glu	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	GLN		GLN		GLX	GLX	GLN	GLN	GLN	his	GLX
	38	GLN	GLN	glu	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	gly		GLN				GLN	GLN	GLN	GLX	tyr
	39	arg	LYS	LYS	#	#	arg	LYS	LYS	LYS	LYS	LYS	LYS		LYS	LYS	LYS	arg		LYS				tyr	tyr	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	gln	PRO	PRO		PRO	PRO	PRO	PRO		PRO				PRO	PRO		PRO	PRO
F	41	GLY	GLY	GLY	#	#	GLY	GLY	GLY	GLY	GLY	GLY	GLY							GLY								
R	42	gln	LYS	glx	LYS	LYS	gln	arg	glx	LYS	LYS	glx																
2	43	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA														
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO														
	45	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys		lys														
	46	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu		VAL														
	47	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU		met														
	48	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE		ILE																
	49	TYR	TYR	ser		TYR	TYR	TYR	TYR	TYR	TYR	TYR		gly														
	50	asp	arg	LYS		asp	asp	asp	glx	asp				asp														
	51	ALA	val	thr	asp	ALA	ALA	ALA		ALA				ALA														
C	52	SER	SER	SER	SER	SER	ala	pha		SER				SER														
D	53	ASN	ASN	ser	ary	ser	ASX	ASN						ser														
R	54	LEU	arg	LEU	LEU	LEU	LEU	LEU		LEU				LEU														
2	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	val	asx	val	val	val						val				ILE										
	59	PRO	PRO	PRO	PRO	glx	PRO	PRO										PRO										
	60	SER	SER	SER	SER	SER		SER						SER				SER										
	61	ARG	ARG	ARG	lys	ARG	ARG	ARG						ARG				ARG										
	62	PHE	PHE	PHE	val	PHE	PHE							gln				PHE										
	63	thr	SER	ala	SER	leu	thr							SER				SER										
	64	GLY	GLY	GLY	GLY	GLY	GLY							GLY				GLY										
	65	SER	SER	SER	SER	SER								SER				SER										
	66	GLY	GLY	GLY	asx	GLY								GLY				GLY										
	67	SER	SER		SER	pha								SER				SER										
	68	GLY	GLY		glu	asx								GLY				GLY										
	69	THR	THR		THR	THR								THR				THR										
	70	asp	asp		asp	lys								asp				GLU										
	71	PRO	PRO		val									pro				PHE										
F	72	pha	THR		THR									ser				THR										
R	73	LEU	pha		val									LEU				LEU										
3	74	THR	THR		asx									ala				THR										
	75	ile	ile		gln									VAL				VAL										
	76	ser	ser		ser									ser				ILE										
	77	ser	ser		asx									ary				ASN										
	78	LEU	LEU		LEU													LEU										
	79	arg	GLN		GLN													GLN										
	80	pro	pro		pro													SER										
	81	glu	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	gln	gln		gln													HIS		gln								
	91	tyr	gly		arg	phe								gly				ASN		tyr								
	92	his	ser		ASP	ASP								glu				ASP		ASP								
	93	asn	his		asp	asp								SER				thr		asp								
C	94	leu	leu		leu	leu								asp				PHE		leu								
D	95	pro	pro		pro	pro								SER				SER		pro								
R	95A	---	---		---	---								---				---		---								
3	95B	---	---		---	---								---				---		---								
	95C	---	---		---	---								---				---		---								
	95D	---	---		---	---								---				---		---								
	95E	---	---		---	---								---				---		---								
	95F	---	---		---	---								---				---		---								
	96	LEU	ARG		TYR	TYR								ARG				TRP		PRO								
	97	THR	THR		THR	THR								THR				THR		THR								
	98	PHE	PHE		LEU	PHE								PHE				PHE		PHE								
	99	GLY	GLY		GLY	GLY								GLY				GLY		GLY								
	100	GLY	GLY		GLY	PRO								GLN				GLN		PRO								
	101	GLY	GLY		GLY	GLY								GLY				GLY		GLY								
	102	THR	THR		THR	THR								THR				THR		THR								
F	103	LYS	LYS		LYS	LYS								LYS				LYS		LYS								
R	104	VAL	VAL		VAL	VAL								VAL				VAL		VAL								
4	105	GLU	GLU		GLU	GLU								GLU				LEU		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* LOM	82 AMYLOID IS305 #	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* H-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	ala	SER	SER	SER
FR1 10	ser	THR	THR	ser	ser	ser	ser	ser	ser	ser		phe	ser	ser	ser	ser	ser	ser	THR		ser	ser	ser	ser	ser
11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU		val	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU
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	ser	ALA	ALA	ALA		ALA	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
14	SER	SER	SER	SER	SER	SER	pro	SER	SER	SER		SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
16	GLY	GLY	GLY	GLY	GLY	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	asy	asy	gly	asy	asy		leu	asy	asy	---	asy	asy	asy	asy	pro	asy	asy	asy	asy	asy	asy	asy	asy	asy	asy
19	val	val	val	val	val	val	val	val	val	---	val	val	val	val	ile	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	len	ILE	ILE	ILE	ILE	ILE
22	THR	THR	ser	THR	THR	THR	THR	THR		---	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR
23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	---	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYD		CYD	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										
CDR1 27C	----	----	----	----	----	----	---	---	---	---	---				val										
27D	----	----	----	----	----	----	---	---	---	---	---				trp										
27E	----	----	----	----	----	----	---	---	---	---	---				ser										
27F	----	----	----	----	----	----	---	---	---	---	---				---										
28	asx	SER	SER	asp	asx	ala	asx	gly	asx	---	asp				asp										
29	ILE	ILE	ILE	ILE	ILE	#	ILE	ILE	ILE	---	ILE				gly										
30	GLY	ser	ser	ser	ser		ser			---	asn				arg										
31		ser		asp		ASN	ASX		pro	---	lys				ser										
32	tyr	ser		tyr		tyr	tyr		tyr	---					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										
FR2 41										---															
42										---															
43										---															
44										---															
45										---															
46										---															
47										---															
48										---															
49										---															
50										LYS															
51										ser															
CDR2 52										---															
53										---															
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71										---															
FR3 72										---															
73										---															
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83										---															
84										---															
85										---															
86										---	TYR														
87										---	TYR														
88										CYS	CYS														
89										glx	gln														
90										glx	gln														
91										glx	tyr														
92										ASX	glu														
93										ala	asn														
CDR3 94										ala	leu														
95										thr	pro														
95A										---	---														
95B										---	---														
95C										---	---														
95D										---	---														
95E										---	---														
95F										---	---														
96										ILE	TYR														
97										PRO															
98										ILE															
99										GLY															
100										GLY															
101										GLY															
102										THR															
FR4 103										LYS															
104										VAL															
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* H-G2a	108 CRA	109 AMYLOID LEP	110* WAG	111 NOE	112 HBJ 1	113 AMYLOID 547	114 WEB	115 HBJ 10	116 LOD	117 BEN	118 MAA	119 GR	120 MUR	121 GM131 'GL #	122 AMYLOID 594	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	---
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	---
	3	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	leu	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
	4	MET	MET	leu	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET		MET	leu	MET	MET		MET	leu	---
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	---
	6	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	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	---
	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	---
FR1	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	LEU	LEU	LEU	LEU	val	LEU		LEU	LEU	LEU	LEU		LEU	LEU	---
	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER				---
	13	ALA	val	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	val					---
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER									---
	15	VAL	VAL	VAL	VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL		VAL								---
	16	GLY	GLY	GLY	GLY	GLY	GLY	arg	GLY	GLY		GLY												---
	17	ASP	glx	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															---
	20	THR	THR	THR	THR	THR		THR	THR															---
	21	ILE	ILE	ILE		ILE	ILE	ILE																---
	22	THR	THR	THR		ala	THR	THR																---
	23		CYS																					---
	24																							---
	25																							---
	26																							---
	27																							---
	27A																							---
	27B																							---
CDR1	27C																							---
	27D																							---
	27E																							---
	27F																							---
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	41																							---
FR2	42																							---
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	51																							---
CDR2	52																							---
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FR3	72																							---
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	76																							---
	77																							---
	78																				LEU			---
	79																				GLN			---
	80																				pro			---
	81																				glu			---
	82																				ASP			---
	83																				PHE			---
	84																				ALA			---
	85																				THR			---
	86																				TYR			---
	87																				TYR			---
	88																				CYS			---
	89																				gln			---
	90																				gln			---
	91																				His			---
	92																				ASP			ASP
	93																				his			ile
	94																				leu			PHE
CDR3	95																				pro			pro
	95A																				---			---
	95B																				---			---
	95C																				---			---
	95D																				---			---
	95E																				---			---
	95F																				---			---
	96																				LEU			GLY
	97																				THR			THR
	98																				PHE			PHE
	99																				GLY			GLY
	100																				GLY			GLN
	101																				GLY			GLY
FR4	102																				THR			THR
	103																				THR			LYS
	104																				VAL			VAL
	105																				GLU			GLU
	106																				MET			ILE
	106A																				---			---
	107																				LYS			LYS
	108																				ARG			ARG
	109																				THR			THR

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (Cont'd)

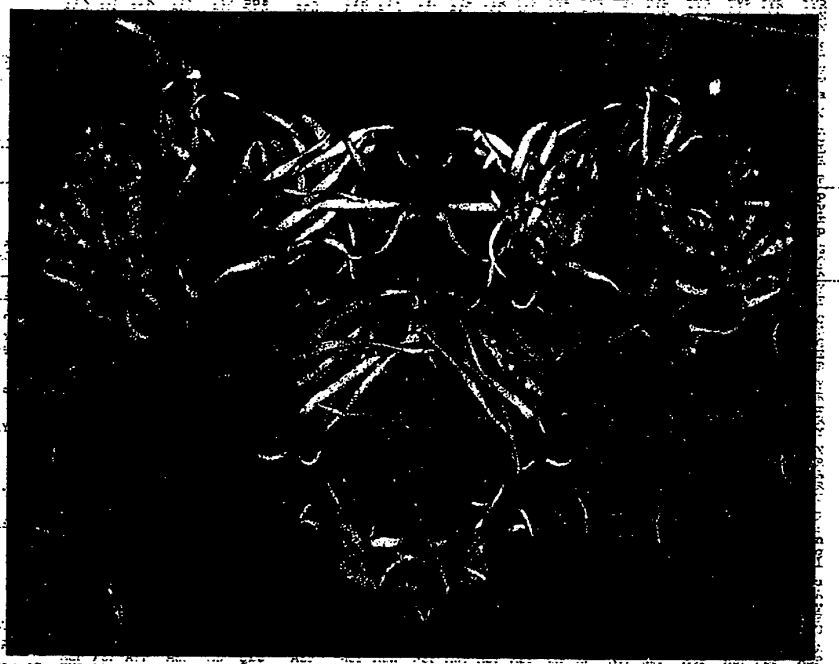
		125 AMYLOID	126 HBJ .6	127 AMYLOID MS #	128 PEN	# OF SEQUENCES	# OF AMINO ACIDS	OCCURRENCES OF MOST COMMON AMINO ACID	VARIABILITY
	0	---	---	---	---	1	1	1 (PCA)	
	1	ASP	ASP	ASP	ASP	125	3,4	122 (ASP), 118 (ASP)	3.1,4.2
	2	ILE	ILE	#	ILE	123	2	120 (ILE)	2.
	3	glu	GLN	GLN	GLN	123	7	116 (GLN), 112 (GLN)	7.4,7.7
	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	#ar				120	5	89 (SER)	6.7
	11	LEU				119	4	111 (LEU)	4.9
	12					118	3	115 (SER)	3.1
	13					117	4	107 (ALA)	4.4
	14					113	6	106 (SER)	6.4
	15					113	3	109 (VAL)	3.1
	16					109	2	108 (GLY)	2.
	17					109	3,4	106 (ASP), 98 (ASP)	3.1,4.0
	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)	4.4
	40					70	3	68 (PRO)	3.1
	41					68	3	54 (GLY)	3.2
FR2	42					60	6	49 (LYS)	7.3
	43					61	4	57 (ALA)	4.3
	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	18 (ALA)	28.
	51					59	5	50 (ALA)	5.9
CDR2	52					58	6	53 (SER)	6.6
	53					57	6,7	26 (SER)	13.,15.
	54					58	2	56 (LEU)	2.1
	55					58	8,9	23 (GLU)	17.,21.
	56					56	8	34 (SER)	13.
	57					57	1	57 (GLY)	1.
	58					58	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)	8.3,11.
	71					54	4	45 (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)	9.1
	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 (PHE)	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					59	11,12	28 (TYR)	23.,25.
	92					60	9	20 (ASN), 19 (+)	27.,28.
	93					60	5	29 (SER)	19.
	94					60	12	15 (LEU)	48.
	95					56	4	44 (PRO)	5.1
CDR3	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.
FR4	103					46	4	38 (LYS)	4.8
	104					45	2	36 (VAL)	2.5
	105					45	6,5	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.

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*[†]

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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
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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

HUMAN KAPPA LIGHT CHAINS

ALIGNMENT RESIDUES	1 HK101 'CL	2 HK 137 'CL	3 VKI- CRE1 'CL	4 HuVH CAMP 'CL	5 VKI-ZI 'CL	6 3D6 'CL	7 V108 'CL	8 Vb 'CL	9 Vb' 'CL	10 HK102 'CL	11 JA12 'CL	12 1H1 '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 A2 'CL	23 A23 'CL
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-21		ASP	ASP	---	---	ASP	ASP	GLU	ASP	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
-19		ARG	ARG	ARG	MET	ARG	ARG	ARG	ARG	MET	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
-17		LEU	LEU	PRO	TRP	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	CYS	GLN	GLN	GLN	GLN	GLN	GLN	LEU	LEU				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
-11		LEU	LEU	LEU	PHE	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
-8	LEU(.95)	LEU	LEU	ALA	ALA	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	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP
-6		PHE	PHE	LEU	ALA	LEU	LEU	LEU	PHE	PHE	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU		VAL	VAL	ILE
-5		PRO	PRO	THR	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO		SER	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	ALA	SER	SER	SER	SER
-2		ARG	ARG	ARG	HIS	ARG	LYS	ARG	ARG	ARG	LYS	LYS	LYS	LYS	LYS	ARG	ARG	ARG	ARG	SER	SER	SER	SER
-1		CYS	CYS	CYS	SER	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	GLY	GLY	GLY	ALA

	24 MG9 'CL	25 HIC (R) 'CL #	26 HAH 14.1 'CL #	27 HAH 14.2 'CL #	28 HAH 16.1 'CL #	29 NOV 'CL #	30 8E10 'CL #	31 TH3 'CL #	32 Humkv 325 'CL	33 EV1-15 'CL	34 IARC/ BL41 'CL	35 Humkv 305 'CL	36 IS1 '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 kv328 'CL #	47 Hum kappa1es 'CL #
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-21		---	---	---	---	---			---	---	---	---	---	---	---	---	---	---	---	---	---	---		
-20		MET	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		
-18		THR	THR	THR	THR	THR			THR	THR	THR	THR	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		
-17		PRO	PRO	PRO	PRO	PRO			PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		
-16		ALA	ALA	ALA	ALA	ALA			ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		
-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		
-9		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-8		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-7		TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		
-6		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-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	GLT	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	ARG	GLY	GLY	GLY

	48 GF4 /1.1 'CL	49 K- EV15 'CL	50 VKAPPA IV GERMLINE 'CL	51 PB17IV 'CL	52 FK-001 'CL #	53 WALKER 'CL	54 DAUDI 'CL	55 VJ1 'CL	56 HK100 'CL	57 Va V2 'CL	58 V2 V14 'CL	59 V14 V52 'CL	60 V52 V55 'CL	61 V55 EV	62 K- EVJK11 'CL	63 PSEUDO VKAPPA 24'CL	# OF SEQUENCES	# OF AMINO ACIDS
-29	---	---	---	---	---	---	---	---	---	---	---	---			---	---		
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-21	---	---	---	---	---	ASP	ASP	---	ASP	ASP	---	---	---	ASP	---	---	17	2
-20	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	52	2
-19	GLU	GLY	VAL	VAL	VAL	ARG	ARG	VAL	ARG	ARG	GLN	GLN	ARG		GLU	ARG	53	6
-18	ALA	SER	LEU	LEU	LEU	VAL	VAL	LEU	VAL	VAL	VAL	VAL	ALA		THR	CYS	53	7
-17	PRO	GLN	GLN	GLN	GLN	PRO	PRO	GLN	PRO	PRO	PRO	PRO	PRO		PRO	SER	54	5
-16	ALA	VAL	THR	THR	THR	ALA	ALA	THR	ALA	ALA	THR	THR	ALA		ALA	PRO	54	5
-15	HIS	HIS	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	HIS	54	4
-14	VAL	LEU	VAL	VAL	VAL	LEU	LEU	VAL	LEU	LEU	LEU	LEU	LEU		LEU	PHE	54	4
-13	LEU	LEU	PHE	PHE	PHE	LEU	LEU	PHE	LEU	LEU	LEU	LEU	LEU		LEU	LEU	54	3
-12	PHE	SER	ILE	ILE	ILE	GLY	GLY	ILE	GLY	GLY	GLY	GLY	GLY	GLY	PHE	GLU	55	6
-11	LEU	PHE	SER	SER	SER	LEU	LEU	SER	LEU	LEU	LEU	LEU	LEU		LEU	LEU	55	3
-10	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	58	1
-9	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	VAL	VAL	LEU	PRO		LEU	VAL	58	5
-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	LEU	ILE	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	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	CYS	SER	GLY	GLY	63	6

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:

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

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Page 170

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

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Page 171

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).

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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

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

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: May 12, 2017
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard Bell *et al.*

Application No.: 15/148,839

Confirmation No.: 3159

Filed: May 6, 2016

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

DECLARATION PURSUANT TO 37 C.F.R. § 1.132 BY DR. LAURAL BOONE

I, Laural S. Boone, J.D., Ph.D., declare the following:

1. I am presently a Senior Director and Patent Counsel at Alexion Pharmaceuticals, Inc., (hereafter, "Alexion") which is located in New Haven, Connecticut. I have been employed in this capacity at Alexion for three years to date. Alexion is the Assignee of the above-referenced patent application. I received a B.A. in Chemistry from Barnard College, a M.S. and Ph.D. in Organic Chemistry from the University of Chicago, and a J.D. from the George Washington University Law School. I also completed a Postdoctoral Fellowship in Cellular and Molecular Biophysics at Rockefeller University. I am registered to practice as a patent attorney before the United States Patent & Trademark Office.

2. I have reviewed the Examiner's request for additional information under 37 C.F.R. § 1.105 and provide the following statements.

3. I have reviewed Exhibits A through F cited by the Examiner on pages 7-8 of the April 4, 2017 Office Action, including each of the 37 documents listed in Exhibit F. These exhibits and the documents listed therein reference 17 different clinical studies using eculizumab that occurred prior to March 16, 2007. Of the 17 studies, 4 are referred to as "extension" studies, which are studies that are appended to a clinical study to prolong the treatment and/or monitoring period of the same patients that participated in the initial study.

4. Of these 17 clinical studies, one that is described in both Exhibits D and F, is entitled "A Randomized, Third-Party-Blind, Placebo-Controlled, Pilot Study of the Effect of h5G1.1-mAb in Patients with Bullous Pemphigoid." Based on my review of communications between Alexion employees and physicians participating in this study, I have determined that only one patient was enrolled in this study, and that the one patient was randomized to receive placebo. Thus, no patient received eculizumab in the course of this study, and the study was terminated.

5. For the remaining 16 clinical studies identified in Exhibits A through F, I have reviewed each of the clinical trial protocols and the following information from the protocols is shown in the table below: the full name of each study (and extension study thereof, if applicable, identified by an "E" or an "X" in the study number), the study number(s), the Exhibit(s) cited by the Examiner on pages 7-8 of the Office Action that each study was described in, and whether the study (including any extension study thereof) included a confidentiality provision (as described in further detail below).

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Study Name[1]	Study Number	Exhibit(s)	Explicit Confidentiality Provision
An Open-Label Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Haemoglobinuria Who Previously Participated in Study C02-001 An Open-Label Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Hemoglobinuria Who Previously Participated in Study E02-001	C02-001 E02-001 X03-001	F	Yes
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 (TRIUMPH)	C04-001	A[2], F	Yes
Safety in Hemolytic PNH Patients Treated with Eculizumab: A Multi-Center Open-Label Research Design Study (SHEPHERD)	C04-002	A, C, F	Yes
A Phase III, Open-Label, Extension Study of Eculizumab in Patients with Transfusion Dependent, Hemolytic Paroxysmal Nocturnal Hemoglobinuria (PNH) Who Have Participated in the TRIUMPH (C04-001), SHEPHERD (C04-002) or X03-001 Studies	E05-001	A, F	Yes
A Randomized, Double-Blind, Placebo-Controlled Pilot Study of the Effect of h5G1.1-mAb on Dermatomyositis Patients	C99-006	B, D[3], F	Yes

[1] Although some of these studies may not have occurred in the United States and thus are not available to give rise to public use under 35 U.S.C. § 102(b), all of the trials are included in this table (regardless of where they occurred).

[2] Of the studies referenced in Exhibit A, studies 41-43, 45-46, 52-54, 56-58 and 60 are all indicated as having been "received" or "started" *after* March 16, 2007, *i.e.*, the date eculizumab was first approved for commercial marketing and sale. Thus, only studies started before the marketing approval, identified as numbers 44, 47-51, 55, and 59 in Exhibit A, are included in this table.

[3] Item 445382 referenced in the Examiner's question regarding the Kaplan reference (Exhibit D) does not refer to an actual study. However, to address any potential questions the Examiner has with respect to

Study Name[1]	Study Number	Exhibit(s)	Explicit Confidentiality Provision
Phase I Study of h5G1.1-mAb in Patients with Rheumatoid Arthritis	C97-001	F	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis Patients Receiving Methotrexate	C99-001	D, F	Yes
A Phase IIB Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide	C01-004	D, F	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy An Open Label Study of the Long-Term Safety of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy	C99-004 E99-004	D, E, F	Yes
Phase I Study of h5G1.1-mAb in Patients with Systemic Lupus Erythematosus	C97-002	D, F	Yes
A Randomized, Double-Blind, Placebo-Controlled Pilot Study of the Effect of h5G1.1-mAb on Psoriatic Patients	C99-007	D, F	Yes
The Paroxysmal Nocturnal Hemoglobinuria Early Access Treatment Protocol (EMBRACE)	C06-002	F	Yes
PNH Registry	M07-001	A, F	Yes

Exhibit D, I reviewed the Kaplan reference in its entirety, and the clinical studies described therein that used eculizumab are also included in this table.

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6. I have reviewed an alignment of the sequence of eculizumab that is listed in the Biologic License Application for Soliris®(eculizumab) ("BLA") with the heavy and light chain sequences of SEQ ID NOs: 2 and 4 of the parent of the pending application, PCT/US2007/006606 (published as WO 2007/106585). I have concluded that the antibody (eculizumab) used in each of the studies included in the above table contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4. However, the protocols used to conduct those studies did not inform either doctors or patients of the sequences.

7. As indicated in the table above, in the course of conducting each of the clinical trials listed, participating physicians and other participants in the study were required to maintain Alexion's confidential and/or proprietary information in strict confidence. For example, these individuals were required to maintain the confidentiality of the clinical trial protocols themselves, as each protocol was marked "Confidential." These individuals were also bound by explicit confidentiality provisions in the protocol. The following language from the protocol for study number C04-001, which refers to the Phase III TRIUMPH study, provides a representative example of the confidentiality requirements set forth directly within these clinical study protocols (emphasis added):

> "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."

8. As another representative example, the protocol for the study in dermatomyositis (study number C02-002) stated:

"THIS CONFIDENTIAL INFORMATION ABOUT AN INVESTIGATIONAL DRUG IS PROVIDED FOR THE EXCLUSIVE USE OF INVESTIGATORS OF THIS DRUG AND IS SUBJECT TO RECALL AT ANY TIME. THE INFORMATION IN THIS DOCUMENT MAY NOT BE DISCLOSED UNLESS SUCH DISCLOSURE IS REQUIRED BY FEDERAL OR STATE LAW OR REGULATIONS SUBJECT TO THE FOREGOING. THIS INFORMATION MAY BE DISCLOSED ONLY TO THOSE PERSONS INVOLVED IN THE STUDY WHO HAVE A NEED TO KNOW WITH THE OBLIGATION NOT TO FURTHER DISSEMINATE THIS INFORMATION. THESE RESTRICTIONS ON DISCLOSURE WILL APPLY EQUALLY TO ALL FUTURE ORAL OR WRITTEN INFORMATION SUPPLIED TO YOU BY ALEXION, WHICH IS DESIGNATED AS 'PRIVILEGED' OR 'CONFIDENTIAL'."

9. All confidentiality provisions in the protocols for the studies listed in the table above were substantively identical to these representative confidentiality provisions from studies C04-001 and C02-002.

10. In addition, each clinical trial protocol that I reviewed also required strict control over the physical drug product (eculizumab). For example, the clinical trial protocol for the TRIUMPH study described the secure storage and inventory and disposal requirements of each participating physician and study site as shown below:

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

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."

11. All of the protocols that I reviewed for the studies listed in the above table included similar (if not identical) drug storage and accountability provisions, except for the PNH Registry protocol (the last entry in the table above). The PNH Registry was not a study in which eculizumab was administered to any patients, but was a registry for all participating patients with PNH either treated or untreated. More particularly, it was a "non-interventional study designed to collect post-marketing pharmacovigilance and effectiveness data on patients treated with Soliris® (eculizumab)" and to "collect information on the progression of disease in the Soliris treated and untreated PNH patient population." Accordingly, the protocol for the PNH Registry did not contain drug storage or accountability provisions. The PNH Registry protocol did, however, include strict confidentiality provisions as indicated in the table above, *e.g.*, requiring all physicians to "agree to maintain the confidentiality of all information received or developed in connection with [the] protocol."

12. To participate in each clinical trial listed in the table above, each physician had to sign the protocol for the trial, committing to adhere to the respective clinical trial protocol's provisions. For example, the TRIUMPH study protocol, just prior to the physician's signature line, states:

> "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."

13. All of the other protocols that I reviewed similarly required each physician (or investigator) to agree to conduct the study in accordance with the protocol.

14. In response to the Examiner's Request (G), based on the BLA approval letter that I reviewed from the Food and Drug Administration Department of Health & Human Services to Alexion regarding eculizumab, the antibody having the heavy chain described by SEQ ID NO: 2 and the light chain described by SEQ ID NO: 4 was first approved for commercial marketing and sale on March 16, 2007.

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.

Laural S. Boone 11 MAY 2017

Laural S. Boone, J.D., Ph.D. Date

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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

Confirmation No.: 2558

Filed: September 9, 2016

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

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 <u>not</u> 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 <u>fourteen</u> 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
```

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------------CH1-------------------------------|Hinge-
FPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCV
FPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGP

-Hinge--|-----------------CH2------------------
ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
PCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP

---------CH2-----------------------------------------
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC

--------CH2-------|-------------CH3---------------
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG

---------------------------CH3---------------------
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV

---------CH3-------------
FSCSVMHEALHNHYTQKSLSLSLGK
FSCSVMHEALHNHYTQKSLSLSLGK
```

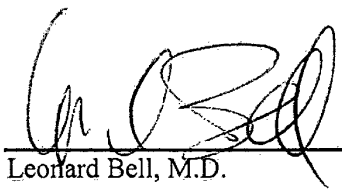
6. Also, the functional properties of eculizumab and the antibody described in the Thomas reference are very different. For example, eculizumab does not bind as strongly to Fcγ receptors as the Thomas antibody. Binding to the Fcγ receptors initiates and amplifies immune responses that are undesirable 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

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
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Questions about this communication ?
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Date
31.07.2017

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466 / 3167888

Applicant/Proprietor
Alexion Pharmaceuticals, Inc.

You are hereby informed that a decision on your request of 10.07.2017 for correction under Rule 139 EPC will be taken during examination proceedings by the competent Examining Division (Guidelines for Examination in the EPO, A-V, 3).

For the publication of the patent application according to Article 93 EPC the documents will be used as originally filed. If the request for correction has been filed before the technical preparations for publication pursuant to Rule 67 EPC have been completed, a reference to the request will be published on the front page.

Receiving Section

Europäisches Patentamt • European Patent Office • Office européen des brevets

Registered letter
EPO Form 1177 12.07

MB50214

Europäisches Patentamt
European Patent Office
Office européen des brevets

Application No.: 16195355.9

Substantive examination has started on 08.11.17 (see Notice from the European Patent Office dated 29.01.2013, OJ EPO 2013, 153).

For the Examining Division

EPO - Munich
102
09. Nov. 2017

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T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Vossius Ref.: M2367 OPP(EP)

Appeal Number T1551/12-3.3.01
EP 07 75 3249.7
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP

EP 16 17 2335.8 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/2

EP 16 19 5355.9 - 1466
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Vossius Ref.: P2913 EP/3

EP 16 20 1244.7 - 1410
Alexion Pharmaceuticals, Inc.
Vossius Ref.: R1249 EP/3

München, November 9, 2017
WA/FLG/MUA

We herewith resign from representation of the five above-identified matters.

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It is respectfully requested that this change is registered in the official Register.

Dr. Joachim Wachenfeld
European Patent Attorney

Samsung Bioepis Exhibit No. 1028
Page 189

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Application No.	Ref.	Date
16 195 355.9 - 1466	P2913 EP/3	17.11.2017
Applicant: Alexion Pharmaceuticals, Inc.		

Communication pursuant to Article 94(3) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(2) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 126(2) and 131(2) and (4) EPC. One set of amendments to the description, claims and drawings is to be filed within the said period on separate sheets (R. 50(1) EPC).

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the Examining Division requesting that you correct this deficiency (R. 137(4) EPC).

In case you withdraw the application within the above-mentioned period, the examination fee will be refunded at a rate of 50% (Art. 11 (b) RFees).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Art. 94(4) EPC).

Registered letter

Collura, Alessandra
Primary Examiner
For the Examining Division

Enclosure(s): 4 page/s reasons (Form 2906)
XP002262113

Registered letter
EPO Form 2001 11.16TRI

The examination is being carried out on the **following application documents**

Description, Pages

1-43, 45-55	as originally filed			
44	received on	10-07-2017	with letter of	10-07-2017

Sequence listings, SEQ ID NO

1-10	received on	10-07-2017	with letter of	10-07-2017

Claims, Numbers

1-3	as originally filed

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 following document is cited by the Examiner. A copy of the document is annexed to the communication and the numbering will be adhered to in the rest of the procedure.

D7 THOMAS T C ET AL: "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv",
MOLECULAR IMMUNOLOGY, PERGAMON, GB,
vol. 33, no. 17-18, 1 January 1996 (1996-01-01), pages 1389-1401, XP002262113,
ISSN: 0161-5890, DOI: 10.1016/S0161-5890(96)00078-8

1. Articles 76(1) and 123(2) EPC

1.1 The objection raised in the European Preliminary Opinion (ESOP) under Article 76(1) EPC is presently maintained.

As already pointed out, the last paragraph on page 5, puts into relation sequence 2 with sequence 4 and not with sequences 8, 9 and 10.

The sequences 8, 9 **or** 10 of the heavy chain were put in relation with sequences 5, 6 **or** 7 of the light chain in a specific embodiment disclosed on page 5, lines 20-25.

No other passage of the application appears to disclose the subject-matter of present claim 1, in which the sequences 8, 9 and 10 have to be all part of the light chain.

It has to be noted by the applicant that the embodiments in which the heavy chain is characterised by sequences 5, 6 or 7 and the light chain by sequences 8, 9 **or** 10, actually include a plurality of embodiments which originate from the possibility of combining different alternative heavy chains (sequences 5, 6 or 7) with different light chains (sequences 8, 9 or 10).

Nowhere in the application as filed was the embodiment selected from these plurality of embodiments, i.e. the combination of sequences 8, 9 and 10, combined with the specific sequence 2.

1.2 With the letter dated 10.07.2017, the applicant filed amended sequences 3 and 4, in which the leader peptide has been deleted, and provided an explanation of the reason why the indicated amendments should be considered as the correction of an obvious mistake under Rule 139 EPC. He also enclosed a declaration by Bruce Andrien as technical expert.

The examining division has carefully considered the arguments of the applicant, but came to the conclusion that it cannot be excluded that the sequences 3 and 4 as originally filed were actually those for which protection was sought. No indication in the original application is present of the fact that sequences 3 and 4 did not have to include the leader peptide.

2. Article 54 EPC

As already objected in the European Search Opinion, the present application is considered not to meet the requirements of Article 54 EPC, because the subject-matter of the claims 1-3 is not new over the disclosure of D1-D4.

The applicant replied to this objection by saying that before the filing date of the parent/present application, i.e March 15, 2007, the sequence of eculizumab was not disclosed.

The arguments of the applicant are not considered to be correct for the following reasons.

All the cited prior art documents D1-D5 refer to eculizumab as a product available on the market. None of them contains an indication of the fact that the sequence of the antibody was secret or that any of the inventor of the patent D1 or of the members of the research group working on the studies published in D2 and D3 were bound to confidentiality with respect to the sequence of the antibody.

Therefore, the sequence of eculizumab was available to public since anybody skilled in the art could have performed a sequence listing of eculizumab with the knowledge available in the art.

D7 already discloses most of the sequence of the light and heavy chains of 5G1.1 (see Figure 1) and explains how the sequence was found. 5G1.1 is eculizumab (see D4).

Moreover, D6 teaches that eculizumanb was entered in the Registry Database on 14.02.1999.

For the reasons given above, the examining division concludes that the structure of eculizumab was available to the public well before the date of filing of the present application and that, as already pointed out in the ESOP the subject-matter of claims is anticipated by the disclosures of D1-D5 teaching eculizumab for use in the treatment of paroxysmal nocturnal hemoglobinuria by intravenous administration and according to the dosage regimen indicated in the present claims.

3. Article 56 EPC

The objection raised in the ESOP concerning inventive step (see point 3 of the communication) is presently maintained.

4. Article 83 EPC

In the description of the invention (see page 5 last paragraph), the applicant states that eculizumab is a specific embodiment of the described invention. The application also discloses examples in which eculizumab is tested. The complete structure of eculizumac has however not been disclosed in the application as filed. At most sequences 2 and 4 for the light and the heavy chain respectively have been said to be included in the sequence of eculizumab, but no indication is provided of the fact that the antibody contains also other sequences in the light and heavy chain.

In his letter dated 10.07.2017, the applicant argues that the structure of eculizumab had not been disclosed before the filing date of the present application.

The obvious consequence of this statement is that the skilled reader, at the filing date of the present application, did not know which antibody was actually tested and corresponded to the name "eculizumab".

Should the applicant therefore argue that the structure of the antibody was not known before the fining of the present application, than the application would be insufficiently disclosed.

If, on the contrary, the name "eculizumab" was known to indicate an antibody available to the public before the filing date of the present application, then the subject-matter of the claims would be not novel (see point 2 above).

5. Procedure

It is not at present apparent which part of the application could serve as a basis for a new, allowable claim. Should the applicant nevertheless regard some particular matter as patentable, an independent claim should be filed taking account of Rule 43(1) EPC. The applicant should also indicate how the subject-matter of the new claim differs from the state of the art and the significance thereof.

Care should be taken not to violate the requirement s of Article 123(2) EPC.

Europäisches Patentamt
European Patent Office
Office européen des brevets

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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
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Date
20.11.17

Reference	Application No./Patent No.
	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication of amended entries concerning the representative (R. 143(1)(h) EPC)

As requested, for the above-mentioned European patent application / European patent the entries concerning the representative have been amended as follows:

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The amendment will be recorded in the Register of European Patents.

For the Examining Division

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Patents – Trademarks – Designs

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December 12, 2017

Third Party Observations

Application Number	16 195 355.9 - 1466
Publication Number	EP 3 167 888
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Applicant	Alexion Pharmaceuticals, Inc.
Our file reference	AMG17203ADV-EP888

Third Party Observations

Amgen Inc. would like to bring the following to the Examining Division's attention:

I. According to Alexion's own submissions, eculizumab and its structure were known in the prior art.

Alexion has submitted in the brief dated July 10, 2017 with regard to the present patent application that D1 to D5 are not novelty-destroying, because only the name, not the structure of eculizumab was known in the prior art before the priority date.

This is in stark contrast to actions and statements made by Alexion in the past:

I.1 Previous patents and patent applications filed by Alexion

Alexion has filed and obtained a significant number of other European patents with one or more claims directed to the use of "eculizumab," e.g., EP2380907B1, EP1988882B1, EP1755674B1, EP2061810B1, EP1720571B9, and also filed various patent applications, all predating the

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Partnerschaftsregister des AG München
PR-Nr. 1278

present application, some with priority dates as early as 2004. A table of patents and patent applications is submitted herewith as **D8**. Of course, there are numerous further patents of Alexion on eculizumab in the USA and other countries. Alexion has thus obtained significant patent protection on the use of eculizumab prior to the filing date of the present application.

I.2 Alexion stated that eculizumab was available

I.2.1 Alexion also explicitly stated that eculizumab was publicly available before the presently relevant date, and accordingly, that the earlier patents were sufficiently disclosed. For example, in the submission dated December 30, 2013, attached herewith as **D9**, in opposition proceedings against EP 1720571, Alexion stated on page 14:

> **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.

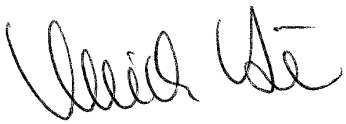
Thus, according to the present applicant, the sequence of eculizumab was publicly available on 14 February 1999, i.e., long before the filing date of the present application on 15 March 2007 or the claimed priority date of 15 March 2006.

I.3 Furthermore, in light of Alexion's past actions, Alexion's declarations in the present case have to be considered to contravene against the generally accepted legal principle of good faith. In particular, in this case, the principle of *venire contra factum proprium* makes these declarations inadmissible.

II. The subject-matter of the claims is not novel over D1-D5

It logically follows that the subject matter of the claims lacks novelty over **D1** to **D5**, and for this reason, as well as other reasons, the application has to be refused.

Respectfully,



Dr. H. Ulrich Dörries

UD/MO/KS

Enclosures

D8 table of Alexion European patents and patent applications on eculizumab predating the present application

D9 submission of Alexion dated December 30, 2013 in opposition proceedings against EP 1720571 of Alexion

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Europäisches Patentamt
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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	AMG17203-AV-888

	Description of document	Original file name	Assigned file name
1	Citation for the limitation procedure	AMG17203ADV-EP888_D8.pdf	LIMDOC-1.pdf
2	Citations filed by a third party	AMG17203ADV-EP888_D9.pdf	TDOC-1.pdf
3	Observations by third parties (Art. 115 EPC)	AMG17203ADV-EP888_TPO.pdf	TIPA1-1.pdf

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Munich, Germany**
Date:	**12 December 2017**
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D9

VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

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EPO - Munich
75
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

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generalisation of the specific contents of the Examples and that in particular the following essential features of the Examples would have been omitted in claims 1 and 5:

(i) the PNH patients are transfusion-dependent and hemolytic

(ii) the meaning of transfusion dependency in the context of the application

(iii) the infusion time of approx. 30 min, and

(iv) the occurrence of the breakthrough between weeks 12 and 64.

1.2.4. With regard to feature (4) the Opponent further alleges that the term "600 mg each week for four weeks" only requires the administration of 600 mg once per week for four weeks, while the Examples would require that the 600 mg are administered at a time interval of exactly 7 days (i.e. weekly intervals) for four weeks.

1.2.5. Finally, the Opponent believes that it would not be clearly supported in the Examples whether the dosages according to features (5) and (6) were intravenously administered. The route of administration would not have been specified in the Examples.

1.3. <u>The new claims have a clear and unambiguous basis in the application as filed.</u>

1.3.1. Granted claim 1 reads:

> "Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule:
> (i) 600 mg each week for four weeks;
> (ii) 900 mg 1 week later;
> (iii) 900 mg on a biweekly basis; and thereafter
> (iv) 900 mg every 12 days,
> wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)."

As will be detailed herein below, granted claim 1 and consequently also granted claim 5 has a clear and unambiguous basis in the application as filed, because

(i) all features of these claims can be directly and unambiguously derived from the Examples of the application as filed, and

(ii) no technically essential feature of the examples has been omitted or changed.

In the following we will provide a clear and unambiguous basis for each of features (1) to (8) as classified by the Opponent.

1.3.2. *Feature (1) - use of eculizumab*

Eculizumab is the antibody which is used in the examples of the application as filed; see page 32, line 14 reading

> "The specific antibody used in the study was eculizumab."

Thus - in contrast to the allegations of the opponent - no selection from a list is required in order arrive at the anti-C5 antibody which is eculizumab. Hence, feature (1) is directly and unambiguously supported by the Examples of the application as filed.

1.3.3. *Feature (2) - treatment of a patient afflicted with PNH*

The Examples of the application as filed at page 32 start to read:

> "Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months." (emphasis added)

Hence - contrary to the assertions of the opponent - no selection from a list is required in order to define the patient to be treated as patient being afflicted with PNH.

The Opponent further considers that the PNH patient has to be further defined as (i) being hemolytic and (ii) having a history of four or more transfusions within twelve months whereas these features are not reflected by the claim.

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(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)

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As regards requirement (1) of the above-described three-point test, we note that nothing in the application as filed conveys the impression that only PNH patients being transfusion-dependent can be treated by eculizumab.

In view of requirement (2) we note that administration of eculizumab prevents hemolytic events by blocking the complement cascade. Since all PNH patients suffer from hemolytic anemia, all PNH patients will benefit from the claimed use of eculizumab. It is of further note that the skilled person immediately understands from the Examples of the application as filed that all PNH patients showing a complement breakthrough should be treated by 900 mg of eculizumab every 12 days. A requirement of a certain transfusion history for the treatment to be successful is not apparent.

With regard to requirement (3) Patentee asserts that all PNH patients and not only transfusion dependent PNH patients may exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii). In more detail, the Patentee has indicated that, to the best of its knowledge, the ability to show breakthrough appears to be independent of the severity of the disease. Instead, breakthrough is related to how rapidly eculizumab is cleared from the circulation. Some patients seem to clear eculizumab at a higher rate than other patients. The severity of the disease - which may be expressed at least in part by the blood transfusion history of a PNH patient - has therefore no technical effect on the other features of granted claims 1 and 5.

It is thus our firm believe that the PNH patient in claims 1 and 5 neither has to be further defined as (i) being hemolytic nor as (ii) having a history of four or more transfusions within twelve months in order to render these claims compliant with Article 123(2) EPC.

1.3.4. *Feature (3) - administered intravenously*

In the Examples on page 32, lines 12-16 it is stated:

> "Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty

> minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis." (emphasis added)

Hence, in the Examples eculizumab is administered intravenously. Again - contrary to the statements of the opponent - no selection from any list in the specification is required in order to define that eculizumab is administered intravenously.

The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered intravenously makes no technical sense. As the Opposition Division knows, an application has to be read with a mind willing to understand; see, e.g., decision T 190/99. Also the prior art cited by the opponent itself shows that eculizumab is routinely administered intravenously. For example, it is stated in D4 that

> "Patients received 600mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week". (emphasis added)

Essentially corresponding statements can be found in D3 and D6.

Granted claims 1 and 5 refer to intravenous administration and not intravenous infusion. The skilled person knows, though, that an intravenous administration is always by a "push" - which is a one-time, rapid injection of the medication into the bloodstream – or by an "infusion" - which is a slow drip of medication into the vein over a period of time. The technical effect is in both cases that eculizumab is delivered in the blood stream. The only difference is the time frame in which the complete dose of eculizumab is administered. However, the time is not characterized as being essential in the application as filed. The indication of the time is also certainly not indispensable in order to work the invention, and varying the infusion time does not require a modification of the other features of the claim. Hence, in connection with the omittance of the intravenous time the criteria of the "three-point test" developed in T 331/87 are clearly met. Omittance of the infusion time of approx. 30 minutes does not constitute a violation of Article 123(2) EPC.

1.3.5. *Feature (7) - breakthrough in serum hemolytic activity*

The study described in the Examples of the application as filed has been initially carried out for 12 weeks and then has been extended to 64 weeks (see, for example, Table 1 or Figure 3 of the application as filed). The breakthrough in 2 out of 11 PNH patients has been observed between weeks 12 and 64 of the study (see Figure 4 of the application as filed).

It is not essential with respect to the claimed dosage regimen when a PNH patient has a complement breakthrough. The essential step is to increase the dosage from 900mg/14d to 900mg/12d once a complement breakthrough is observed. As it is described in the Examples at page 38, lines 15-17, the complement blockade was permanently restored by shortening the interval of administration - although both PNH patients likely did not show the complement breakthrough at the same time point during the 64-weeks study.

Hence, it is not technically necessary to define in granted clams 1 and 5 at which particular point in time a breakthrough is observed in a PNH patient to be treated.

1.3.6. *Features (4) to (6) and (8)*

The fact that also the dosages according to features (5) and (6) were administered intravenously has been discussed herein above in connection with feature (3).

The Opponent's interpretation of the wording of feature (4) is a mere play on words and should not attract any attention.

Beyond these points the Opponent apparently does not contest that features (4) to (6) and (8) as such can be clearly and unambiguously derived from the Examples of the application as filed. Having said that, the Opponent alleges that these features would only find support in connection with further features of the Examples. These further allegedly omitted features have been discussed herein above in connection with features (1) to (3) and (7).

As is evident from the above discussion in connection with features (1) to (3) and (7) these features do not violate Article 123(2) EPC.

1.3.7. The Opponent does not consider that the features in the dependent claims give raise to any further violation of Article 123(2) EPC. Hence, all of the granted claims comply with Article 123(2) EPC.

2. PRIORITY (Article 87 EPC)

2.1. For essentially the same reasons put forward by the Opponent in section 4 entitled "added subject-matter", the Opponent believes that the subject-matter of the claims cannot be directly and unambiguously derived from the priority application.

The Opponent concludes that the effective date of the granted claims is not the priority date of February 3, 2004 but the international filing date of February 3, 2005.

2.2. As will be shown in the following the relevant contents in the Examples of the application as filed - which form a clear and unambiguous basis for the granted claims - are also comprised in the priority document US 10/771,552; compare page 30, lines 17-18 of the priority document and page 32, lines 16-20 of the application as filed.

In more detail, page 30 of US 10/771,552 reads:

> 5 **EXAMPLES**
>
> Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusion within twelve months. The

and

> Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis. The first twelve weeks of the study constituted the pilot study. All patients participated in an extension study conducted to a total of 48 weeks.

These two passages give rise to priority for features (1) to (6).

Page 30 of US 10/771,552 reads

> 0.001). As seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST. At the next dose, symptoms resolved (Fig 5) and reduction from 900mg every 14 days to 900mg every 12 days resulted in a regain of complement control which was maintained for over 9 months in both patients. This patient shows a 24 hour resolution of

and page 34 of US 10/771,552 reads

> eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, Figure 4).

These two passages give rise to priority for features (7) and (8).

Otherwise arguments in favour of clear and unambiguous support in the application as filed as detailed in section 1, *supra* apply *mutatis mutandis* for entitlement of priority.

Hence, the subject-matter of granted claims 1 and 5 has also a clear and unambiguous basis in the priority application. The priority claim is valid.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opponent considers that the claims lack novelty in view of D1.

3.2. D1 has been published on February 5, 2004 and hence 2 days after the priority date of the patent. D1 is not prior art because the claims are entitled to the priority date; see section 2, *supra*.

4. INVENTIVE STEP (Article 100(a) and 56 EPC)

4.1. The Opponent alleges that the granted claims lack inventive step in view of D5 considered alone or in combination with any one of D2, D3, D4 or D6. The Opponent also contends that the granted claims are no inventive in view of any one of D2, D3, D4 or D6.

4.2. D5 may be considered as closest prior art in the assessment of inventive step of the granted claims. D5 describes:

> "We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year. [...] Two patients experienced transient breakthroughs in complement activity associated with LDH, hemoglobinuria and mild symptoms; these resolves immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in dosing interval." (emphasis added)

Details on the 3-months pilot study are, for example, described in D3, D4 and D6 (see page 53, left column). Hence, D5 (in combination with any one of D3, D4 and D6) could be seen as describing that 2 out of 11 PNH patients exhibited a complement breakthrough when treated under a dosage regimen similar, but not identical to that recited in the granted claims. D5 furthermore teaches that the complement breakthrough can be permanently prevented by a slight decrease in dosing interval.

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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.

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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

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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)

Publication Number	Title	Priority Date	Publication Date	Claims reciting "eculizumab"
EP2380907B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	2006-09-05 2007-09-05	2016-11-30	6. The antibody or antigen-binding fragment thereof for use according to claim 5, wherein said antibody is eculizumab.
EP1988882B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2006-03-02 2007-03-02	2014-12-17	17. The anti-C5 antibody for use according to claim 13, wherein said whole antibody is eculizumab. 37. The use according to claim 34, wherein said antibody fragment is pexelizumab or said whole antibody is eculizumab.
EP1755674B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16	2014-11-19 (WO 2005/110481 published 11/24/2005)	15. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 10, wherein said whole antibody is eculizumab.
EP2061810B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	2006-09-05 2007-09-05	2014-11-12	5. The antibody for use according to claim 1 or 2, wherein said antibody is eculizumab.
EP1720571B9	METHOD OF TREATING HEMOLYTIC DISEASE	2004-02-03 2005-02-03	2012-10-24 (A2 published 11-15-2006; WO 2005/074607A2 published 8-18-2005)	1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule: (i) 600 mg each week for four weeks; (ii) 900 mg 1 week later; (iii) 900 mg on a biweekly basis; and thereafter (iv) 900 mg every 12 days, wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii). (All eight claims recite "eculizumab")
EP3056218A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16 2005-05-16	2016-08-17	10. The antibody to C5 according to claim 9 wherein said whole antibody is eculizumab.
EP2910253A1	METHOD OF TREATING HEMOLYTIC DISEASE	2004-02-03 2005-02-03 2005-02-03	2015-08-26	2. The anti-C5 antibody for use according to claim 1, wherein the anti-C5 antibody is eculizumab. 9. The use according to claim 8, wherein the anti-C5 antibody is eculizumab.
EP2815767A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16	2014-12-24	6. The composition according to claim 3, wherein the whole antibody is eculizumab.
EP2089058A2	METHODS OF TREATING HEMOLYTIC ANEMIA	2006-11-08 2007-11-08	2009-08-19	18. The method of claim 15, wherein said antibody is eculizumab. 34. The method of claim 32, wherein said antibody is eculizumab. 40. The method of claim 37, wherein said antibody is eculizumab. 59. The method of claim 56, wherein said antibody is eculizumab. 75. The method of claim 73, wherein said antibody is eculizumab. 100. The method of claim 97, wherein said antibody is eculizumab. 116. The method of claim 114, wherein said antibody is eculizumab. 122. The method of claim 119, wherein said antibody is eculizumab. 141. The method of claim 138, wherein said antibody is eculizumab. 157. The method of claim 155, wherein said antibody is eculizumab. 163. The method of claim 160, wherein said antibody is eculizumab.
EP2013235A1	METHODS FOR THE TREATMENT OF MUSCULAR DYSTROPHY ASSOCIATED WITH DYSFERLIN-DEFICIENCY	2006-05-01	2009-01-14	6. The method of claim 1, wherein said antibody is eculizumab.
EP1881993A2	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES	2005-05-11 2006-05-10	2008-01-30	7. The method of claim 1 , wherein the antibody is eculizumab or pexelizumab.

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	5868642
Application number	EP16195355.9
Date of receipt	12 December 2017
Receiving Office	European Patent Office, The Hague
Your reference	AMG17203-AV-888
Applicant	All applicants as on file
Documents submitted	package-data.xml epf1038.pdf (1 p.) TDOC-1.pdf\AMG17203ADV-EP888_D9.pdf (16 p.) ep-sfd-request.xml LIMDOC-1.pdf\AMG17203ADV-EP888_D8.pdf (1 p.) TIPA1-1.pdf\AMG17203ADV-EP888_TPO.pdf (3 p.)
Submitted by	CN=Hans Doerries 13689
Method of submission	Online
Date and time receipt generated	12 December 2017, 15:12 (CET)
Message Digest	1F:D1:F0:D2:2D:26:3B:94:97:54:65:66:E0:66:62:02:B8:6D:59:E6

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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ROYAUME UNI

Date
18-12-2017

Reference	Application No./Patent No.
	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication pursuant to Rule 114(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115 EPC).

Under Rule 114(2) EPC you may comment on the observations.

For the Examining Division

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Date
18-12-2017

Reference	Application No./Patent No.
	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Acknowledgment of receipt of observations by third parties (Article 115 EPC)

Receipt of your letter dated 12.12.2017 is hereby acknowledged.

Under Article 115 EPC you will not be a party to the proceedings before the European Patent Office.

☐ In your letter the following documents are mentioned which were not enclosed, and which are not available in the EPO:

☐ The third party observations have not been filed in an official language of the EPO (R. 114(1) EPC).

You are requested to file copy(ies) and/or translation(s) in one of the official EPO languages within **two months** of notification of this communication if they are to be taken into account.

For the Examining Division

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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

16 January 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

In response to the Communication pursuant to Article 94(3) EPC dated 17 November 2017, please replace the claims currently on file with the new claims attached herewith. Please also replace description page 5 with new description page 5 attached herewith.

To assist the Examiner, a tracked changes version of the claims and a tracked changes version of description page 5 showing the amendments being made are also attached.

Also attached are: (i) a copy of a letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007; (ii) a copy of a press release made by Alexion Pharmaceuticals on 16 March 2007; (iii) a Declaration of Stephen Saxe; and (iv) an Experimental Report.

Amendments

Claims 1 and 2 have been combined as new claim 1. An obvious error in referring to the whole of SEQ ID NO: 4 in the definition of the light chain of the antibody has been corrected.

A corresponding correction has been made at page 5 of the description.

Correction of Obvious Error

In the response of 10 July 2017 to the Search Opinion, correction of the sequence listing was requested. In the corrected sequence listing, the leader peptide was deleted from SEQ ID NO: 3 and SEQ ID NO: 4. The Examining Division has determined

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that this correction of the sequence listing is not allowable under Rule 139 EPC because it considers that there is no indication in the original application that SEQ ID NO: 3 and SEQ ID NO: 4 did not have to include the leader peptide.

Rather than correcting the sequence listing, we now request correction of the statements in the description that:

> "*In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3*"; and
>
> "*In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ ID NO: 4*".

It would be immediately evident to the skilled person that an antibody light chain variable region could not consist of SEQ ID NO: 3 and that the light chain of an antibody could not consist of SEQ ID NO: 4.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "*The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1)*

Reading the present application, the skilled person would immediately recognise, based on his common general knowledge, that each of SEQ ID NO: 3 and SEQ ID NO: 4 contain a leader sequence as explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien.

The skilled person would also know that leader sequences are not present in an antibody ready for binding an antigen because these sequences are cleaved off during antibody maturation and production in cells. Therefore, it would be immediately evident to the skilled person that the references at page 5 of the description to an antibody comprising a light chain variable region consisting of SEQ ID NO: 3 and an antibody comprising a light chain consisting of SEQ ID NO: 4 represent an error.

As previously explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien, using his common general knowledge, the skilled person would have no difficulty determining which of the amino acids shown in SEQ ID NO: 3 and SEQ ID NO: 4 correspond to a leader peptide.

Therefore, the corrections being made to page 5 of the description to refer to the start and end positions of the light chain variable region and the light chain of the antibody would immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC.

It follows that the reference to the start and end positions of the mature part of the light chain sequence in SEQ ID NO: 4 in claim 1 complies with the requirements of Article 123(2) EPC.

Novelty

Claim 1 is novel because there was no public prior use of eculizumab before the filing date of the present application and no prior art document contains an enabling disclosure of eculizumab.

Eculizumab was not available on the market at the filing date

Contrary to the assertion in the Examination Report, none of D1-D5 indicate that eculizumab was a product available on the market prior to the filing date of 16 March 2007.

D1 is a US patent application which describes therapy trials to evaluate the effects of an anti-C5 antibody on the rare disease PNH and symptoms associated therewith. At page 9, paragraph [0082] of D1 it is stated that "*the specific anti-C5 antibody used in the study was eculizumab*". However, D1 does not say where eculizumab was obtained from, and does not indicate that eculizumab was commercially marketed or otherwise available to the public.

D2 is an abstract describing a clinical trial showing that eculizumab appears to be the first effective therapy for haemolysis in PNH. D2 discloses that "*eculizumab is a human monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). Like D1, D2 neither indicates from where eculizumab was obtained, nor does it indicate that eculizumab was commercially marketed or otherwise available to the public.

D3 is a paper describing the effect of eculizumab on haemolysis and transfusion requirements in patients with PNH. D3 states that: "*eculizumab is a recombinant humanised monoclonal antibody that was designed to block the activation of terminal complement components*". Again, no indication as to where eculizumab was obtained from is given, and there is no indication that eculizumab was commercially marketed or otherwise available to the public.

D5 is a report of an interview with Anita Hill relating to the use of eculizumab for the treatment of PNH. D5 does not provide any information as to how eculizumab may be obtained, nor does it suggest that eculizumab was commercially marketed or otherwise available to the public.

D4 is a paper describing eculizumab. D4 states that "*eculizumab (5G1.1) is a recombinant, fully humanised monoclonal antibody that inhibits the activation of*

terminal complement components" (D4, Abstract, first sentence). D4 indicates that the source of eculizumab is Alexion Pharmaceuticals, Inc. (US) (D4 page 6-7, right column, third paragraph), but it does not indicate that eculizumab was commercially marketed or otherwise available to the public.

Eculizumab was, in fact, not commercially marketed or otherwise available to the public at the publication dates of any of these references. As explicitly stated in the Declaration of Laural Boone, a Senior Director and Patent Counsellor at Alexion Pharmaceuticals, Inc. filed with our response of 10 July 2017, eculizumab was first approved for commercial marketing and sale on 16 March 2007, which is the day after the filing date of the present application (see paragraph 14 of the Boone Declaration).

The Boone Declaration is further supported by the attached letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007, licensing Alexion Pharmaceuticals to manufacture the product eculizumab. The attached press release made by Alexion Pharmaceuticals on 16 March 2017 announcing that it had, that day, received marketing approval from the U.S. Food and Drug Administration (FDA) for eculizumab.

Therefore, it is a matter of fact that eculizumab was not a product available on the market at the filing date of the present application.

The structure of eculizumab was not publically known at the filing date

None of the cited documents disclose the full amino acid sequence of eculizumab.

Eculizumab is an antibody that does not correspond to any naturally occurring isotypes. Rather, eculizumab has a unique, non-naturally occurring, protein-engineered full heavy chain (including the CH1-hinge-CH2-CH3 regions). This unique heavy chain sequence is not disclosed in the prior art.

D7 provides sequence information for an anti-C5 designated antibody h5G1.1. Eculizumab is derived from h5G1.1. However, D7 only discloses the heavy and light chain variable region (VH and VL) sequences of the antibody (see Figure 1 of D7). Significantly, D7 does not disclose the full, uniquely-engineered heavy chain sequence of eculizumab. There is also an amino acid difference between the light chain variable region sequence shown in D7 and the light chain variable region sequence of eculizumab. A glutamine (Gln/Q) residue is present at position 60 of SEQ ID NO: 4 but an arginine residue (Arg/R) is present at this position in the light chain variable region sequence shown in D7 (position 38 of the mature sequence of "h5G1.1 VLC" in Figure 1 of D7).

Thus, D7 does not disclose an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

D1, D2 and D5 describe eculizumab by name only; they do not disclose the structure of eculizumab.

D3 and D4 each refer to Thomas et al. (1996) Mol. Immunol. 33: 1389-401 (reference 15 in D3) which is cited as D7 in the Examination Report of 17 November 2017. As

discussed above, D7 does not disclose the full heavy and light chain sequences of eculizumab. Therefore, D3 and D4 do not enable the skilled person to make eculizumab.

The Examining Division notes that D6 indicates that eculizumab was entered in the CAS registry database on 14 February 1999. However, D6 is a copy of the CAS registry database on 6 March 2017. D6 cannot therefore be used as evidence that SEQ ID NO: 2 and SEQ ID NO: 4 were available to the public ten years earlier at the 15 March 2007 filing date.

The sequence information for eculizumab submitted to the CAS Registry on 14 February 1999 was incorrect. The correct sequence information for eculizumab was not entered on the registry database until May 2018, which is after the filing date of the present application.

The attached Declaration of Stephen Saxe, and its annexes, show that the sequences for CAS Registry Number 219685-50-4 as shown in D6 were not available in the CAS Registry database until May 2009. In particular, Annex C, the email sent from Lois Bishop at CAS Client Services to Stephen Saxe on 1 May 2009 confirms that changes were made to the sequence for CAS Registry Number 219685-50-4 that would appear on STN a couple of days after that email was sent. Annex B, an email and letter sent from Stephen Saxe to the CAS, shows that the CAS Registry Number 219685-50-4 heavy chain sequence in the database prior to May 2009 was 650 amino acids in length, whereas SEQ ID NO: 2 is a 448 amino acid sequence, and that a single error was corrected in the light chain sequence (see paragraph 3 of the letter and point 3 of the Saxe Declaration).

Thus, the heavy chain sequence in the CAS database at the filing date of the present application contained hundreds of amino acids that are not present in SEQ ID NO: 2 and there is one amino acid difference between the light chain sequence in the CAS database at the filing date of the present application and residues 23 to 214 of SEQ ID NO: 4. Therefore, D6 is not evidence that an antibody comprising the heavy chain sequence shown in SEQ ID NO: 2 and the light chain sequence shown in residues 23 to 214 of SEQ ID NO: 4 was available to the public at the filing date of the present application.

In addition, as set out in the Declaration of Laural Boone, the heavy and light chain sequences of eculizumab (SEQ ID NOs: 2 and residues 23 to 214 of SEQ ID NO: 4 in the present application) were not made available to doctors or patients participating in any of the trials using eculizumab prior to at least 16 March 2007 (see paragraphs 6 and 7 of the Boone Declaration).

Thus, the full amino acid sequences of the heavy and light chain sequences of eculizumab were not disclosed in any of the cited documents.

The skilled person would not have been able to analyse the structure of eculizumab and reproduce it

In G1/92, the Enlarged Board of Appeal concluded that the chemical composition of a product is state of the art when the product as such is available to the public and can be analysed and reproduced by the skilled person. However, none of the studies

using eculizumab as described in D1-D5 made eculizumab available to the public such that the skilled person would have been able to analyse the structure of eculizumab and reproduce it.

As set out in the Declaration of Laural Boone, all physicians and participants in the eculizumab trials were required to maintain Alexion's confidential and/or proprietary information in strict confidence (see paragraphs 6 and 7 of the Boone Declaration). The clinical trials required strict controls to be placed over the use of eculizumab as explained in paragraphs 10 and 11 of the Boone Declaration. In particular, all eculizumab not administered to patients in the trials had to be accounted for (paragraph 10 of Boone Declaration).

Therefore, the skilled person would not have been able to obtain and analyse eculizumab to determine its structure, and would not have been able to reproduce eculizumab at the filing date of the present application.

Third Party Observations

The third party observations filed on 12 December 2018 are merely circumstantial and do not provide any evidence that eculizumab was publically available at the filing date of the present invention.

Summary

Although reports of trials describing eculizumab were available at the filing date of the present application, eculizumab was not made publically available until after the filing date. Eculizumab was not on the market at the filing date; the full structure of eculizumab was not published; and the skilled person would not have been able to analyse eculizumab and reproduce it.

Accordingly, there was no public prior use, public availability or enabling disclosure of eculizumab at the filing date. Claim 1 is therefore novel.

Inventive Step

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive. In particular, SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any information about the structure of eculizumab. Accordingly, it would not have been obvious to the skilled person from any of these documents to protein engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2. Accordingly, claim 1 involves an inventive step.

Sufficiency

The application provides a sufficient description of the claimed invention. SEQ ID NO: 2 is the complete heavy chain amino acid sequence of eculizumab, and residues 23 to 214 of SEQ ID NO: 4 represent the complete light chain amino acid sequence of eculizumab. Therefore, the complete structure of eculizumab is disclosed in the application.

In contrast, as discussed above, the full heavy and light chain sequences of eculizumab were not disclosed before the filing date of the present application. Thus, the skilled person reading any of D1 to D5 did not know the structure of the antibody that was actually tested and corresponded to the name “eculizumab.” However, in view of the inclusion of the full heavy chain sequence of SEQ ID NO: 2 and the full light chain sequence in SEQ ID NO: 4 (residues 23 to 214), the present application provides the first enabling disclosure of the antibody eculizumab.

Accordingly, the requirements of Article 83 EPC are met by the present application.

Concluding Remarks

It is believed that this application is now in an allowable form and the Examiner is asked to reconsider it favourably. If, however, there are any matters which remain outstanding, the Examiner is requested to issue a further Examination Report or telephone the undersigned. Merely as a precaution, in the event that the Examiner is minded to reject the application without first issuing a further Examination Report, oral proceedings are requested.

In addition, if the Examiner is minded to issue a Rule 71(3) Communication but wishes to propose amendments to the claims or description, it is requested that the Examiner telephones the undersigned to discuss this before the Rule 71(3) Communication is issued. I look forward to hearing from the Examiner in due course.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

Europäisches Patentamt
European Patent Office
Office européen des brevets

Letter accompanying subsequently filed items

Representative:

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Amended description (clean copy)	Amended pg 5 (clean) N411599EP-B PXT.pdf	DESC-1.pdf
2	Amended claims (clean copy)	Amended claims (clean) N411599EP-B PXT.pdf	CLMS-1.pdf
3	Amended description with annotations	Amended pg5 (tracked) N411599EP-B PXT.pdf	DESC-HWA-1.pdf
4	Amended claims with annotations	Amended claims (tracked) N411599EP-B PXT.pdf	CLMS-HWA-1.pdf
5	Reply to examination report	Response to OA N411599EP-B PXT.pdf	EXRE3-1.pdf
6	Reply to examination report	Dept of Health & Human Services letter N411599EP-B PXT.pdf	EXRE3-2.pdf
7	Reply to examination report	Alexion Pharmaceuticals press release N411599EP-B PXT.pdf	EXRE3-3.pdf
8	Reply to examination report	Saxe Declaration re CAS Eculizumab Sequence N411599EP-B PXT.pdf	EXRE3-4.pdf
9	Reply to examination report	Experimental Report N411599EP-B PXT.pdf	EXRE3-5.pdf

	Payment	
1	Method of payment	Not specified

Signatures

Place:

Date: **16 January 2018**

Signed by: **Elizabeth Davenport 55598**

Association:	**J A Kemp**
Representative name:	**DAVENPORT, Elizabeth**
Capacity:	**(Representative)**

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months. In certain embodiments, said patient has aplastic anemia or myelodysplastic syndrome.

In certain embodiments, said antibody that binds C5 or an active antibody fragment thereof is administered in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the single unit dosage form is lyophilized. In certain embodiments, the single unit dosage form is a sterile solution. In certain embodiments, the single unit dosage form is a preservative free formulation. In certain embodiments, the 300 mg single-use dosage form comprises 30 ml of a 10 mg/ml sterile, preservative free solution.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises an altered constant region, wherein said antibody or antigen-binding fragment exhibits decreased effector function relative to an anti-CDCPl antibody with a native constant region. In certain embodiments, decreased effector function comprises one or more properties of the following group: a) decreased antibody-dependent cell-mediated cytotoxicity (ADCC), and b) decreased complement dependent cytotoxicity (CDC), compared to an anti-CDCPl antibody with a native constant region. in certain embodiments, the altered constant region comprises a G2/G4 construct in place of the G1 domain.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:?, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of residues 23 to 109 of SEQ ID NO: 3. In certain embodiments, the pharmaceutical composition comprises eculizumab. In certain embodiments, the pharmaceutical composition comprises pexelizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of residues 23 to 214 of SEQ ID NO: 4.

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CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

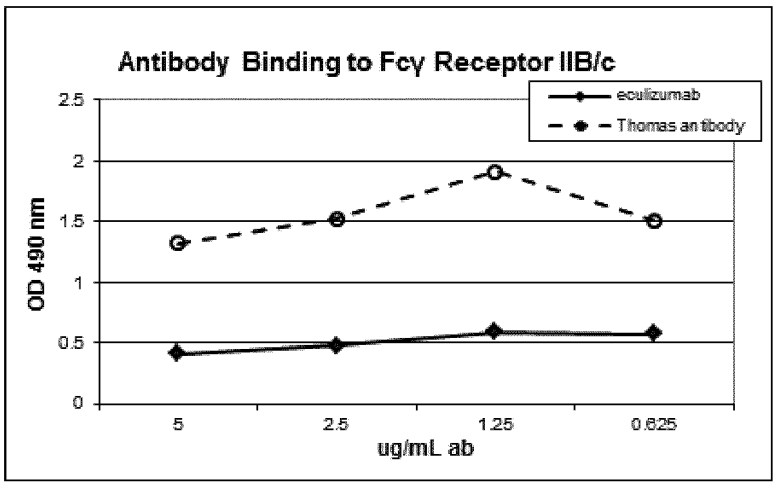
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Experimental Report

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.

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

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answers @ cas. org
send new seqs, reference old order #
sf disulfide bond change - let them know,
Iris Bishop 614-447-3600, x 2863

Saxe, Stephen

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

Dear Sir or Madam,

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

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

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****

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
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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:

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. 1028
Page 241

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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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.

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

http://www.alexionpharm.com

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News Provided by COMTEX

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,

Karen Weiss for

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

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

months. In certain embodiments, said patient has aplastic anemia or myelodysplastic syndrome.

In certain embodiments, said antibody that binds C5 or an active antibody fragment thereof is administered in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the single unit dosage form is lyophilized. In certain embodiments, the single unit dosage form is a sterile solution. In certain embodiments, the single unit dosage form is a preservative free formulation. In certain embodiments, the 300 mg single-use dosage form comprises 30 ml of a 10 mg/ml sterile, preservative free solution.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises an altered constant region, wherein said antibody or antigen-binding fragment exhibits decreased effector function relative to an anti-CDCPl antibody with a native constant region. In certain embodiments, decreased effector function comprises one or more properties of the following group: a) decreased antibody-dependent cell-mediated cytotoxicity (ADCC), and b) decreased complement dependent cytotoxicity (CDC), compared to an anti-CDCPl antibody with a native constant region. in certain embodiments, the altered constant region comprises a G2/G4 construct in place of the G1 domain.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:?, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of residues 23 to 109 of SEQ ID NO: 3. In certain embodiments, the pharmaceutical composition comprises eculizumab. In certain embodiments, the pharmaceutical composition comprises pexelizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of residues 23 to 214 of SEQ ID NO: 4.

Samsung Bioepis Exhibit No. 1028
Page 254

~~New EP divisional application~~
~~based on EP 11 00 1632.6~~
~~Alexion Pharmaceuticals, Inc.~~
~~Our Ref.: P2913 EP/3~~

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: ~~2~~ and a light chain ~~comprising SEQ ID NOS: 8, 9 and 10.~~

~~2. The antibody of claim 1 comprising a light chain~~ consisting of residues 23 to 214 of SEQ ID NO: 4.

~~3~~2. A pharmaceutical composition comprising the antibody of claim 1 ~~or 2~~.

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Page 255

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European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	5964149
Application number	EP16195355.9
Date of receipt	16 January 2018
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file

Documents submitted	
package-data.xml	ep-sfd-request.xml
epf1038.pdf (2 p.)	DESC-1.pdf\Amended pg 5 (clean) N411599EP-B PXT.pdf (1 p.)
CLMS-1.pdf\Amended claims (clean) N411599EP-B PXT.pdf (1 p.)	DESC-HWA-1.pdf\Amended pg5 (tracked) N411599EP-B PXT.pdf (1 p.)
CLMS-HWA-1.pdf\Amended claims (tracked) N411599EP-B PXT.pdf (1 p.)	EXRE3-1.pdf\Response to OA N411599EP-B PXT.pdf (8 p.)
EXRE3-2.pdf\Dept of Health & Human Services letter N411599EP-B PXT.pdf (8 p.)	EXRE3-3.pdf\Alexion Pharmaceuticals press release N411599EP-B PXT.pdf (3 p.)
EXRE3-4.pdf\Saxe Declaration re CAS Eculizumab Sequence N411599EP-B PXT.pdf (8 p.)	EXRE3-5.pdf\Experimental Report N411599EP-B PXT.pdf (2 p.)

Submitted by	CN=Elizabeth Davenport 55598
Method of submission	Online
Date and time receipt generated	16 January 2018, 16:06 (CET)

Message Digest	AA:35:BB:A0:3B:E1:BE:8E:6C:14:45:E9:64:B3:F7:98:86:FF:D7:C4

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

6 March 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

Further to our letter of 16 January 2018 responding to the Examination Report dated 17 November 2018, it has come to our attention that there is an error in one of the dates in our letter.

The second sentence in the third paragraph at page 5 of our letter of 16 January 2018 should be read as follows:

> "*the correct sequence information for eculizumab was not entered on the registry database until May 2009~~2018~~, which is after the filing date of the present application*".

The correct date of May 2009 is used in the fourth paragraph at page 5 of our letter of 16 January 2018.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

PATENT ATTORNEYS • TRADE MARK ATTORNEYS
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A list of our partners is available at our principal place of business at the address above. Regulated by IPREG

Europäisches Patentamt
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Office européen des brevets

Letter accompanying subsequently filed items

Representative:

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Reply to examination report	Correction of error in response N411599EP-B PXT.PDF	EXRE3-1.PDF

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Oxford, GB**
Date:	**06 March 2018**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	6114273
Application number	EP16195355.9
Date of receipt	06 March 2018
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file
Documents submitted	package-data.xml epf1038.pdf (1 p.) ep-sfd-request.xml EXRE3-1.PDF\Correction of error in response N411599EP-B PXT.PDF (1 p.)
Submitted by	CN=Pamela Tuxworth 23470
Method of submission	Online
Date and time receipt generated	06 March 2018, 17:37 (CET)
Message Digest	C7:A7:D3:FC:46:24:13:7C:F3:43:CD:54:0F:56:2E:E8:21:88:44:5A

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Europäisches Patentamt
European Patent Office
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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

Request:

- Enquiry as to the processing of the file

	Description of document	Original file name	Assigned file name
1	Enquiry as to the processing of the file – electronically generated		EPF1012.PDF

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Oxford, GB**
Date:	**19 April 2018**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

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Enquiry as to the processing of the file

I/we hereby enquire when the EPO will deliver the (supplementary) European search report / next communication[1]

for application/patent	16195355.9

[1] OJ EPO 2016, A66

Signatures

Place:	**Oxford, GB**
Date:	**19 April 2018**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	6248515	
Application number	EP16195355.9	
Date of receipt	19 April 2018	
Receiving Office	European Patent Office, The Hague	
Your reference	N411599EP-B	
Applicant	All applicants as on file	
Documents submitted	package-data.xml epf1038.pdf (1 p.)	ep-sfd-request.xml epf1012.pdf (1 p.)
Submitted by	CN=Pamela Tuxworth 23470	
Method of submission	Online	
Date and time receipt generated	19 April 2018, 11:59 (CEST)	
Message Digest	03:DA:9E:AA:7C:99:C1:0D:29:D8:9B:51:50:D0:BD:A3:A5:CB:25:A8	

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
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J A Kemp
14 South Square
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London WC1R 5JJ
ROYAUME UNI

Date
07.05.2018

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Summons to attend oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to attend oral proceedings arranged in connection with the above-mentioned European patent application.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will not be public, will take place before the Examining Division

on 05.02.19 at 09.00 hrs at the EPO,
PschorrHöfe, Bayerstr. 34, 80335 Munich

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC, see also OJ EPO 2008, 471).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to Special edition No. 3 OJ EPO 2007, L.1, concerning the filing of authorisations for company employees and lawyers acting as representatives before the EPO.

The final date for making written submissions and/or amendments (R. 116 EPC) is 04.01.19.

The actual room number as well as the waiting room numbers will be given to you by the porter in the foyer at the above EPO address.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at one of the porters' lodges in the PschorrHöfe, the parking ticket will be revoked.

Please note that the oral proceedings will always take place as scheduled unless you are otherwise informed. The EPO will generally not send a reminder or a further communication prior to the date of oral proceedings.

1st Examiner:	2nd Examiner:	Chairman:
Collura A	Terenzi C	Orlando M

Registered letter with advice of delivery
EPO Form 2008 01.18 [ORAL03=9999] (02/05/18)

to EPO postal service: 02.05.18

Samsung Bioepis Exhibit No. 1028
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For the Examining Division

Annexes:
Confirmation of receipt (Form 2936)
Communication (EPO Form 2906)

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Page 265

Europäisches Patentamt
European Patent Office
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European Patent Office
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GERMANY
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Fax: +49 89 2399 4465

Application No.: 16 195 355.9

Preparation for oral proceedings - Instructions to Support Service

Oral proceedings are to be held in connection with the above patent application

1. The matters to be discussed are set out in the annex (Form 2906)

2. Dispatch the summons using Form 2008/2310 and Form 2906 for the parties to attend on:

Day 05.02.2019 Time 09:00

Archibus

Conference Room 9999 booked

ORAL 01, 02, 03 and 05 coded

02.05.18 m
Date Initials

2.1 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

1 month(s)

before the date of the oral proceedings
(transfer to Form 2008.1 / 2310.1)

2.2 Encode ORAL(04)

coded

02.05.18 m
Date Initials

2.3 Dispatch Form 2008.7 / 2310.7 to division

02.05.18 m
Date Initials

Europäisches Patentamt
European Patent Office
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European Patent Office
80298 MUNICH
GERMANY
Tel: +49 89 2399 0
Fax: +49 89 2399 4465

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. 1028
Page 267

The examination is being carried out on the **following application documents**

Description, Pages

1-4, 6-43, 45-55	as originally filed			
44	received on	10-07-2017	with letter of	10-07-2017
5	filed in electronic form on			16-01-2018

Sequence listings, SEQ ID NO

1-10	received on	10-07-2017	with letter of	10-07-2017

Claims, Numbers

1, 2	filed in electronic form on	16-01-2018

Drawings, Sheets

1/4-4/4	as originally filed

Reference is made to the documents already cited in the course of the procedure.

Moreover, the applicant should be aware of documents D8 and D9 filed by Third Parties on 12.12.17 which may be discussed in the course of the procedure and of WO2005/074607 (D10) which is the publication pamphlet of EP1720571, referred to in D9.

1. Articles 76(1) and 123(2) EPC

The present application does not meet the requirements of Article 76(1) EPC for the following reasons.

The applicant amended page 5 of the description of application as filed (which is the same as the description of th e parent application), and introduced a limitation to the length of the sequence of the indicated SEQ ID NO:3 and SEQ ID NO:4.

The applicant also amended the claims and introduced the same limitation to the length of the SEQ ID NO:4 in present claim 1.

In particular, instead of the full length of the sequences 3 and 4 provided in the application, the specific embodiments disclosed on page 5 and claimed in claim 1 are presently directed to:

(i) an antibody having a heavy chain variable region consisting of SEQ ID NO:1 and a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3; and

(ii) an antibody (as claimed) comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4.

In his letter dated 16.01.2018, the applicant explained that the amendments that he had carried out were to be considered the correction of an obvious mistake which met the requirements of Rule 139 EPC.

As already stated in the official communication from the examining division dated 17.11.17, it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and in the application as originally filed were actually those for which protection was sought.

Moreover, in the original and parent application no disclosure was present of the fact that sequences 3 and 4 did not have to include the leader peptide.

For the reasons given above, the examining division concludes that the subject-matter of the present application does not meet the requirements of Articles 76(1) and 123(2) EPC.

2. Article 54 EPC

2.1 Eculizumab is considered to have been available to the public before the filing date of the present application

a) In the official communication dated 17.11.17, the examining division expressed its opinion on the fact that, since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the product had not been available for public use or that the working groups using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals, it had to be concluded that the structure of the antibody was available to the public at the time of filing of the present application.

Moreover, the examining division pointed out that any of the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. Reference was also made to D7, which disclosed most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explained how the sequence was found.

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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European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
14 South Square
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London WC1R 5JJ
ROYAUME UNI

Date: 07.05.2018

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2008

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately**:

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0) 70 340-3016)**
- oder per Post / or by post / ou par courrier.

Empfangen am / Received on / Reçu le :

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Empfangsberechtigter/authorised recipient/
le destinataire ou la personne dûment mandatée

Rücksende-Adresse / Return address / Adresse de retour
(Umschlag / envelope / enveloppe ISO C4 / DL / C6/C5 / C6)

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DEUTSCHLAND

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Samsung Bioepis Exhibit No. 1028
Page 274

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GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
14 South Square
Gray's Inn
London WC1R 5JJ
ROYAUME UNI

Date: 07.05.2018

Reference	Application No./Patent No.
N L1159QEP-B	16195355.9 - 1112 / 3167888
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2008

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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
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15 August 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT/cxe

Thank you for the Summons to Oral Proceedings dated 7 May 2018. Our further submissions on the points raised by the Examining Division in the Summons are set out below. We attach the following documents:

- a second Declaration of Dr Bruce Andrien dated 15 August 2018;
- an extract from Alberts et al (1994);
- a copy of the Opposition statement filed against EP1720571 (D11);
- a redacted version of the representative clinical trial protocol C04-001; and
- a redacted version of the representative clinical trial protocol C02-002.

The comments below address each and every issue identified by the Examining Division in the Summons to Oral Proceedings. Given the early filing of this response in advance of the 5 February 2019 date set for Oral Proceedings, the Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback, so that the Applicant has time to address any outstanding issues before the date set for filing final written submissions.

Articles 76(1) and 123(2) EPC

The amendment complies with Rule 139 EPC because a skilled person would have immediately recognised that the applicant intended to claim only the mature antibody.

The amendment of the description and claims to refer to an antibody having a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 and to an antibody having a light chain variable region consisting of residues 23-214 of SEQ ID NO: 4 meets the requirements of Articles 76(1) and 123(2) EPC.

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The Examining Division considers that this amendment is not a correction that complies with Rule 139 EPC because

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application as originally filed were actually those for which protection was sought."*

However, the skilled person would have immediately recognised that the applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> *"The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1; emphasis added)*

It was common knowledge that immature heavy and light chains included a signal peptide at the N-terminus, and a skilled person could have readily identified the signal peptide in SEQ ID NOs: 3 and 4.

As addressed in the Andrien Declaration filed on 10 July 2017, it was common general knowledge in the art at the filing date of the application that a signal peptide is present at the N-terminus of immature heavy and light chain polypeptides. Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) (Exhibits B to E filed with the Andrien Declaration on 10 July 2017), which is a database of biological sequences containing sequence alignments, is evidence of the skilled person's common general knowledge and shows the signal peptides of various human light chains.

Page 1 from Kabat *et al.* shows the 22-residue signal peptides of human kappa light chains. The first three residues are predominantly Met Asp Met and the last two residues are predominantly Arg Cys. In each of SEQ ID NO: 3 and SEQ ID NO: 4 of the patent, the first three residues are Met Asp Met and residues 21 and 22 are Arg Cys. Therefore, the skilled person would recognise that SEQ ID NO: 3 and SEQ ID NO: 4 are the amino acid sequences of the immature light chain variable region and the light chain, respectively, that contain a signal peptide that is not present in the mature antibody.

Furthermore, the skilled person would know from his common general knowledge that the framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat *et al.* Residues 23

to 26 of SEQ ID NO: 4 are Asp Ile Gln Met, which the skilled person would recognise as being the start of the mature light chain of the disclosed antibody.

It is also part of the common general knowledge of the skilled person that antibodies are produced using cell-based recombinant methods, and not by artificially synthesising the polypeptide chains. The attached second Declaration of Dr Bruce Andrien dated 15 August 2018 refers to the textbook Alberts et al (1994) Molecular Biology of the Cell and explains that antibodies are processed by the producing-cell in the same way as other secreted proteins, and that this processing always results in the N-terminal signal peptide being cleaved off such that it is not present in the mature antibody.

Thus, during antibody production, the light chain polypeptide sequence of SEQ ID NO: 4, or of SEQ ID NO: 3, is processed to remove the signal peptide. Therefore, the skilled person would know based on his common general knowledge that it would not have been the intention of the Applicant to seek protection for an antibody that contains a signal peptide.

Accordingly, the correction to refer to an antibody comprising only the portion of SEQ ID NO: 3 or SEQ ID NO: 4 that reflects the mature antibody is an obvious correction in the sense that it is immediately evident that nothing else would have been intended than what is offered as the correction within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the application as filed.

The corrections being made to page 5 of the description to refer to the portion of the light chain variable region or the light chain that would be found in the antibody would therefore immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC, Article 76(1) EPC and Article 123(2) EPC.

It follows that claim 1, which refers only to the portion of the light chain sequence shown in SEQ ID NO: 4 that is actually found in the mature antibody, also complies with the requirements of Article 76(1) EPC and Article 123(2) EPC.

The EPO has routinely accepted corrections of this exact type.

The European Patent Office has on other cases accepted that correction of applications defining an antibody as comprising heavy and light chain sequences including signal peptides to redefine the antibody as comprising only the mature proteins of the heavy and light chain sequences meets with the requirements of Rule 139 EPC. Two such examples include EP1558648, in which the correction allowed by the Examining Division was unsuccessfully challenged by the Opponent in an Opposition, and EP2581113.

Article 54 EPC

Eculizumab was not available to the public before the filing date of the present application.

The Examining Division has, after considering the arguments provided in relation to the question of whether eculizumab was publicly available at the filing date of the present application, expressed its opinion in paragraphs 1 to 4 of the Summons. Our comments on each of these points are set out below.

1. The fact that Alexion Pharmaceuticals received marketing authorisation for eculizumab from the FDA on 16 March 2007 means that eculizumab was not available on the market at the 15 March 2007 filing date of the present application. This appears to be accepted by the Examining Division. The Examining Division considers, however, that the antibody could still have been available to the public or that its structure could have been known before the priority/filing date of the present application. As discussed below, eculizumab was not publicly available and its structure was not publicly known at the filing date.

2. It is clear from Mr Saxe's Declaration that Alexion Pharmaceuticals inadvertently submitted the incorrect sequence of eculizumab to the Chemical Abstract Services (CAS) such that the sequence entered into the CAS STN database on 14 February 1999 did not contain the heavy and light chain sequences set out in claim 1 of the present application. It is irrelevant whether or not Alexion had any intention of keeping the substance secret after the date of submission to CAS. It is similarly also irrelevant whether anybody to whom the erroneous sequence in CAS would have been obvious could have contacted Alexion Pharmaceuticals to inquire about the correct sequence. Although Mr Saxe corrected the sequence submitted to CAS as soon as he became aware of the error, the fact remains that as of the filing date of the present application, the correct sequence of eculizumab was not available via CAS.

   It is completely alien to the concept of novelty at the EPO that a mistake in a document, the correction of which is not immediately obvious, can be treated as if the document disclosed the correct information. The fact is that the correct sequence was not disclosed in the CAS registry on 14 February 1999. What was *presumably intended to be disclosed* in the CAS registry on 14 February 1999 does not matter for purposes of assessing novelty.

   Not only was an incorrect sequence disclosed on 14 February 1999, but the corrections that would need to be made to the sequences entered on the CAS STN database on 14 February 1999 to arrive at the correct sequence of eculizumab as disclosed in SEQ ID NOs: 2 and 4 of the present application would not have been immediately obvious to the skilled person.

   Firstly, with regard to the light chain sequence, there was a single error in the sequence of the light chain that was submitted in 1999 as explained in the letter of 28 April 2009 from Dr Stephen Saxe to CAS, which letter was enclosed with the Saxe Declaration filed with our response of 16 January 2018. This error was the inclusion of an arginine (R) residue at position 38 of the light chain sequence instead of a glutamine (Q) residue at this position. It would not have been obvious to the skilled person that the inclusion of an arginine residue instead of a glutamine residue was an error. Indeed, as

shown in Figure 1B at page 1392 of D7, the 5G1.1 and h5G1.1 antibodies referred to therein contain an arginine (R) at position 38.

With regard to the heavy chain amino acid sequence, the sequence originally submitted and included in the STN database on 14 February 1999 was a 650 amino acid sequence, whereas the correct sequence has a length of only 448 amino acids. As shown in the annotated version of the incorrect heavy chain sequence originally submitted to CAS, which is included with the Saxe Declaration, the initially submitted sequence contains three blocks of amino acid residues that are not present in the eculizumab sequence. The first two blocks of amino acid sequences are inserted and the third block of amino acids substitutes a shorter stretch of amino acids present in eculizumab but missing in the incorrect sequence originally submitted to CAS. In addition, there are two single amino acids in the constant region of the heavy chain sequence that differ between the originally submitted sequence and the correct sequence. All of the errors in the originally submitted sequence would not have been obvious to the skilled person.

Even if the skilled person had recognised that the heavy chain sequence in the CAS STN database was unusually long for a heavy chain sequence and had turned to Thomas *et al*, 1996 (D7 in the present proceedings), which is the literature paper describing an antibody referred to as h5G1.1, he would have seen that the human IgG4 isotype was chosen to prepare a full length humanised 5G1.1 antibody in D7 (see D7, page 1396, sentence spanning left and right columns and page 1399, right column, lines 24 to 31). The skilled person would not have known that the heavy chain constant region intended to be submitted to CAS is not the IgG4 constant region used in D7.

The amino acid sequence of the IgG4 heavy chain in D7 differs from the heavy chain shown in SEQ ID NO: 2 of the present application and as correctly submitted to CAS in April 2009. The differences are illustrated at pages 2 to 3 of the Declaration of Dr Leonard Bell filed on 10 July 2017. There are 14 amino acid residues that differ between the IgG4 heavy chain constant region sequence and the heavy chain constant region sequence of eculizumab shown in SEQ ID NO: 2 of the present application. As shown in the Bell Declaration, there are four differences in the CH1 domain, six differences in the hinge region and four differences in the CH2 domain. Therefore, even if the skilled person would have turned to D7 to try and determine the correct sequence of eculizumab, D7 would have provided him with a very different sequence than that described in SEQ ID NO: 2.

In the third party observations filed 12 December 2017, attention was drawn to a statement made by the Patentee in the Opposition proceedings against EP1720571 in response to an allegation by the Opponent that the claims of that patent were insufficiently disclosed. A copy of the Opposition statement filed by Pfizer Inc against EP1720571 is attached as D11. It can be seen from Section 7.1 at pages 12 to 13 of this Opposition statement that the Opponent alleged that the complete sequence of eculizumab is not disclosed in U.S. Patent No. 6,355,245 or in Thomas *et al*, *Molecular Immunology*, Vol.33, No.17/18, pages 1389-1401, 1996 (D7 in the present proceedings).

In response to this objection, in paragraph 5.1.2 of the response filed on 30 December 2013, the Patentee stated that:

> *"The Patentee asserts that the sequence for eculizumab was publically available prior to the priority and filing date. A sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry No. 219685-560-4"* [emphasis added].

The copy of the CAS entry No. 219685-50-4 that was attached with the response of 30 December 2013 did not include any sequence information. Only a one page fax dated 04/08/2008 (8 April 2008) from CAS was filed with the response (a copy of which was provided with the third party observations filed on 12 December 2017). As the sequence of eculizumab on file at CAS had not been corrected as of 8 April 2008, and certainly not as of the priority date of the opposed patent, this statement by the Patentee referenced an incorrect sequence of eculizumab and was clearly made in error. It is clear that this erroneous statement was made inadvertently and in good faith. Neither the third party observer nor the Examining Division has pointed to any evidence of bad faith in conjunction with the incorrect statement, and indeed none exists. Further, despite this unfortunate mistaken statement in the Opposition response, the fact remains that the full correct sequence of eculizumab was not publicly disclosed or publicly available before 16 March 2007.

It is also noted that Alexion's statement in the aforementioned opposition response was never challenged because the Opponent had withdrawn the Opposition on 7 October 2013. Nor was Alexion called on by the Opposition Division to provide evidence of the eculizumab sequences published by CAS in 1999. Indeed, sufficiency of disclosure was not further discussed in the Opposition proceedings on EP1720571. The assertion made by Alexion was not relied on in any way in those proceedings, and the statement had no influence on the outcome of the proceedings, in which EP1720571 was revoked for lack of inventive step. Thus, Alexion's mistaken statement amounted to nothing more than a mere assertion that turned out to be erroneous but that had no bearing on the outcome of that case. Alexion obtained no benefit as a result of the inadvertent misstatement.

The third party argued in paragraph I.3 of his observations of 12 December 2017 that:

> *"in light of Alexion's past actions, Alexion's declarations in the present case have to be considered to contravene against the generally accepted legal principle of good faith. In particular, in this case, the principle of venire contra factum proprium makes these declarations inadmissible".*

However, the legal principle of *nemo potest venire contra factum proprium* does not apply in this situation.

The principle of *nemo potest venire contra factum proprium* has been applied by the EPO's legal Boards of Appeal where the EPO has exhibited contradictory behaviour, i.e. where the actions of the EPO have given rise to legitimate expectations which precluded the EPO from later going back on its own previous conduct (see for example, J27/94 and J14/94 summarised in Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 519, paragraph 2.3).

In the present case, the assertion made in the Opposition response was not relied on or considered any further in the Opposition proceedings. It did not affect the outcome of the Opposition proceedings in which the patent was revoked for lack of inventive step. Accordingly, the assertion made in the response to the opposition of EP1720571 does not take precedence over the signed declarations made in connection with the present application, and it does not bind Alexion Pharmaceuticals in any way.

The Examining Division considers that:

> *"Even if it were now purported that Alexion Pharmaceuticals would have never disclosed the correct sequence to anybody who had asked them for it, that would not amount to a valid defence since under the legal principle of "nemo potest venire contra factum proprium" Alexion Pharmaceuticals would be bound to its declared intention to lay the compound open under CAS rules in order to have other patents be regarded as sufficiently disclosed".*

It appears that the Examining Division is attempting to apply the legal principle of *nemo potest venire contra factum proprium* in a different way than that suggested by the third party. Whilst the third party cited this legal principle in order to suggest that all of the declarations filed in the present application should be ignored, the Examining Division appears to consider that this principle would have bound Alexion Pharmaceuticals to disclose the correct sequence of eculizumab to anybody who requested it in order to have "*other patents be regarded as sufficiently disclosed*". It is presumed that the Examining Division is using the term "*other patents*" to refer to the patents and applications listed in D8 filed by the third party.

There are a number of points to be made here. Firstly, the legal principle of *nemo potest venire contra factum proprium* does not oblige a party who has submitted incorrect data to provide the correct data to any third party who requests it. Alexion is also unaware of what "CAS rules" the Examining Division refers to.

Secondly, the Examining Division seems to suggest that even though, in fact, the correct sequence of eculizumab was not disclosed before 16 March 2007, it must be considered to have been effectively disclosed so that the antibody defined using the term "eculizumab" used in previously-filed patents and patent applications listed in D8 is sufficiently disclosed. Clearly, this reasoning is legally flawed. Lack of novelty cannot be established based on an unsupported presumption of sufficiency of an earlier patent. Rather, the issue of novelty solely rests on the factual inquiry of whether or not the

claimed invention was in the public domain prior to the effective filing date. In the present case, it was not. Therefore, the claims are novel.

Moreover, all of the patents and patent applications listed in D8, with the exception of EP1720571 which was revoked for lack of inventive step, refer to eculizumab by name only in dependent claims (see the right column in the Table in D8). The independent claims of these patents and applications refer to anti-C5 antibodies more generally.

There is no doubt that anti-C5 antibodies were sufficiently disclosed in 2004, which is the year of the earliest priority date of any of the patents and patent applications listed in D8. For example, all of the patents and patent applications refer to US Patent 6,355,245 and many refer also to Thomas *et al*, 1996 (D7 in the present proceedings). These documents both disclose heavy and light chain variable region sequences of anti-C5 antibodies, and D7 also discloses a full length humanised IgG4 anti-C5 antibody.

In any event, as stated above, it is of no import whether disclosure of the full correct sequence of eculizumab would have been necessary in order for all of the claims of the earlier patents and patent applications to meet the sufficiency requirements of Article 83 EPC - the *fact* remains that the full correct sequence of eculizumab was *not* disclosed prior to 16 March 2007.

The Examining Division considers that:

> "*The statement from the Applicant acting as Patentee on EP1720571 (see D9, page 14 Item 5.1.2) confirms the fact that he considered the structure of eculizumab to have been disclosed at the filing date of the present application*".

Again, this statement from the Patentee was an inadvertent error made in good faith. The issue of sufficiency was not further discussed in the course of the opposition and the assertion made by the Patentee was not challenged, the Opponent having withdrawn his Opposition on 7 October 2013. In the statement made in connection with the Opposition against EP1720571, Alexion Pharmaceuticals made no reference to SEQ ID NO: 2 or SEQ ID NO: 4, and did not make any admission that these particular sequences were publically available.

Also, the fact noted by the Examining Division that the date of the assertion in D9 (30 December 2013) is later than the date in which the correction of the eculizumab structure was requested to CAS is irrelevant to the discussion of novelty of the present application. The assertion cannot alter what was, or was not, disclosed at an earlier date.

The fact remains that the sequences submitted by Alexion Pharmaceuticals to CAS and entered into the CAS STN database on 14 February 1999 are not the sequences of SEQ ID NOs: 2 and 4 set out in claim 1 of the present application.

3. The Examining Division considers that the

> *"many patent and non-patent literature documents published before the priority date and relating to the use of eculizumab can be regarded as evidence that the compound was well-known and available to the public before the priority date of the patent application".*

However, this position is not in line with the case law. The Boards of Appeal have established that the mention of a compound in a published document does not mean that the compound is available to the public.

In T576/91, the Board of Appeal stated that if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made *"available to the public"* within the meaning of Article 54(2) EPC.

Further, in T128/92 the Board of Appeal stated that for a complex biochemical to be made available to the public, the minimum that would seem to be required for publication was a notice to those in the field that samples of the biochemical could be obtained upon request, and clear evidence of exactly what the biochemical was. (See Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 89, paragraph 3.2.5).

At page 2 paragraph 2.1(a) of the Summons, the Examining Division expresses the opinion that

> *"since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the products had not be available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals, it had to be concluded that the structure of the antibody was available to the public at the time of filing of the present application".*

This conclusion is, however, clearly contrary to the case law discussed above. It cannot be concluded from a publication that an antibody was publicly available based merely on the absence of a statement that the antibody was *not* available. To the contrary, a positive statement to this effect would be required. No such statement is included in any of the cited documents.

The Examining Division has noted that different published patent applications belonging to the Applicant that mention "eculizumab" were available at the priority date of the present application. Such patents and applications are listed in D8 filed by the third party on 12 December 2017. None of these earlier applications, or the references they cite, disclose the full sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

The mere existence of earlier patents and applications that mention "eculizumab" without precisely defining it does not destroy the novelty of

claim 1 of the present application. The present Examining Division is tasked with assessing the novelty of the claims of the present application in light of all of the *factual* information that was available in the prior art, and the fact is that none of the patents and applications listed in D8 disclose an antibody comprising the full heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

4. The Examining Division has requested proof of the confidentiality agreement between the company and each participant/physician involved in the studies described in D1 to D5. Dr Laural Boone, a Senior Director and Patent Counsel at Alexion, stated that she has reviewed the protocols of all of the clinical trials that were carried out with eculizumab prior to 16 March 2007 and found strict confidentiality and drug accountability provisions in each of the protocols. (See the Declaration of Dr Boone filed on 10 July 2017).

The Table at page 3 of Dr Boone's declaration lists all of the trials for which she reviewed the protocols, and paragraphs 7 and 8 of her Declaration provide examples of the confidentiality provisions she found in the protocols. The representative protocols from the studies Dr Boone refers to, C04-001 and C02-002, are now attached in redacted form. The confidentiality provision referred to in paragraph 7 of the Boone Declaration is found in paragraph 11.1 of protocol number C04-001, and the provision referred to in paragraph 8 of the Boone Declaration is found in the final paragraph at page 46 of study number C02-002.

In addition, as paragraph 10 of Dr Boone's declaration makes clear, the protocols also contained strict drug accountability provisions. The provisions referred to in paragraph 10 of Dr Boone's declaration are set out in paragraph 4.6 spanning pages 19 and 20 of protocol number C04-001, and in paragraph 6.6 at page 24 of study number C02-002. These provisions ensured that no study drug (eculizumab) was released to the public, and, as stated in the Boone Declaration, were included in all clinical trial protocols identified in the Declaration. This evidence demonstrates that all physicians and participants in the clinical trials were bound by strict confidentiality and drug accountability provisions so that there was no public disclosure or public availability of eculizumab via the trials.

Moreover, it should be noted that even if the participants and physicians involved in the studies had not had these strict conditions imposed on them by their agreements with Alexion Pharmaceuticals, the structure of eculizumab still would not have been publicly available, because, in accordance with G1/92, the skilled person would not have been able to analyse the structure of eculizumab and reproduce it without an undue burden.

In T2045/09 the Board considered whether the public sale of an antibody made that antibody publicly available in the sense that the skilled person would be able to determine its internal structure and reproduce it without unburden in accordance with G1/92.

In T2045/09, the Board noted that in a document published in 2006, the Authors emphasised that the sequencing of the variable regions of antibodies comprising the CDRs constituted a major scientific challenge and that:

> *"In particular, the unique character of the CDRs renders an alignment and the comparison of the data to already existing antibody sequences in databases impossible. A complete and sufficiently correct amino acid sequence could only be achieved by complementing the data of the Edman sequencing with sequence obtained independently from mass spectrometry".*

In view of the challenge the skilled person would have faced in trying to sequence an antibody in 2006, the Board was not persuaded that the skilled person could have correctly determined the amino acid sequence of the relevant antibody with the knowledge and the technologies available (see Reasons 35 and 36 of the Decision).

In the Summons at page 2, last paragraph, the Examining Division points to the official Communication dated 17 November 2017 in which it

> *"pointed out that any of the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. Reference was also made to D7, which disclosed most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explained how the sequence was found".*

However, at least the uniquely-engineered, non-naturally-occurring portions of eculizumab were not disclosed in D7 and, as explained above as found in T0245/09, obtaining the full sequence of eculizumab from the eculizumab molecule alone would not have been routine in the art at the 15 March 2006 priority date of the present application.

2.2 The Examining Division maintains that the disclosure of D1-D5 teaching eculizumab and its use in the treatment of PNH are considered novelty destroying for the subject matter of the claimed invention. The claims of the present application are, however, novel in view of the disclosure of D1-D5.

Each of D1-D5 refers to eculizumab by name only. None of these documents disclose the heavy and light chain sequences of eculizumab. As discussed in paragraphs 2.1(3) above, the mere mention of a complex biochemical by name in a publication does not enable that complex biochemical to be made or make that complex biochemical available to the public. Therefore, the claims of the patent application are novel over D1-D5.

2.3 The present application is novel over the disclosure of D10. D10 is an earlier patent application of Alexion Pharmaceuticals (WO 2005/074607). This document does not provide an enabling disclosure for an antibody having the heavy and light chain sequences of SEQ ID NOs: 2 and 4.

WO 2005/074607 relates to methods of treating a hemolytic disease by administrating an anti-C5 antibody to a subject having a hemolytic disease (see claim 1 of D10). D10 discloses that:

> *"Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanised Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol.33, No.17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab"* (page 18, lines 12 to 20).

Thus, D10 defines eculizumab by reference to U.S. Patent No. 6,355,245 and Thomas *et al*, 1996 (D7 in the present proceedings). Neither of these documents discloses the sequences of SEQ ID NOs: 2 and 4. Therefore an antibody having the heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4 is novel over D10.

The assertion made by the Applicant as Patentee in the response to the Opposition against EP1720571 derived from D10 cannot change what is disclosed in D10, and, as discussed above, is not an admission that SEQ ID NO:2 and SEQ ID NO: 4 were publically known at the filing date of EP1720571. The fact is that D10 does not disclose an antibody having the mature heavy and light chain sequences of SEQ ID NOs: 2 and 4, and nothing said later can change this. Therefore, the claims of the present application are novel over D10.

In conclusion, none of the prior art documents disclose an antibody having the sequences recited in claim 1, and such an antibody was not publically available in the sense of Article 54(2) EPC. Therefore, claim 1 is novel.

Article 56 EPC

The claims of the present application are novel for the reasons set out above. The claims also involve an inventive step in accordance with Article 56 EPC.

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive.

SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human

IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and it would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any further information about the structure of eculizumab. It would therefore not have been obvious to the skilled person from any of these documents to engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2.

Accordingly, claim 1 involves an inventive step.

Article 83 EPC

Regardless of the conclusions reached with respect to Articles 76(1) EPC, Article 123(2) EPC and Article 54 EPC, the claimed subject matter is sufficiently disclosed in the present application.

Claim 1 is directed to:

> *"An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4."*

It would be within the routine skill of a person skilled in the art to make an antibody having the specified heavy and light chain amino acid sequences.

There is also no doubt that the sequences disclosed in the present application identify the structure of eculizumab. SEQ ID NO: 2 is the amino acid sequence of the full mature heavy chain of eculizumab and the sequence of SEQ ID NO: 4 is the amino acid sequence of the full immature light chain of eculizumab. Therefore, the sequences filed with the original application are those identifying the structure of eculizumab.

For the reasons explained above, the skilled person would know based on his common general knowledge which residues of SEQ ID NO: 4, or of SEQ ID NO: 3, correspond to the signal peptide and which residues correspond to the mature portion of the therapeutic antibody known as eculizumab, i.e. residues 23 to 214 of SEQ ID NO: 4.

Furthermore, in terms of actually producing the eculizumab antibody, a polynucleotide encoding the whole of SEQ ID NO: 4 could be expressed in a cell along with a polynucleotide encoding the heavy chain of eculizumab, i.e. SEQ ID NO: 2. The signal peptide of SEQ ID NO: 4 would be cleaved off during antibody production in the cell, resulting in an antibody whose light chain contains only the mature portion of SEQ ID NO: 4.

Thus, the skilled person would have known from the disclosure of the present application which structure was to be assigned to the therapeutic antibody eculizumab.

Conclusion

The Examining Division is requested to reconsider this application favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. Given the early filing of this response in advance of the 5 February 2019 date set for Oral Proceedings, the Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be allowed without the need for Oral Proceedings. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson



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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

tein translocator. There is now strong evidence for a protein translocator. Normally, the pore in the translocator is plugged with the growing polypeptide chain that is in transit across the membrane; when the nascent chains are experimentally released from the ribosomes with the drug puromycin, however, the pores can be detected by the ion currents that flow through them. Although the pore is large enough to allow the passage of an unfolded polypeptide chain, it closes when the ribosome is removed from the membrane (Figure 12–42). Thus the pore seems to be a dynamic structure, opening when a ribosome with a growing polypeptide chain attaches to the membrane and closing when the ribosome detaches after the synthesis of the protein is completed.

The ER Signal Peptide Is Removed from Most Soluble Proteins After Translocation [31]

We have seen that in chloroplasts and mitochondria the signal peptides are cleaved from the precursor proteins once they have crossed the membrane. Similarly, amino-terminal ER signal peptides are removed by a signal peptidase on the luminal side of the ER membrane. The peptide by itself, however, is not sufficient to signal cleavage by the peptidase; this requires an adjacent cleavage site that is specifically recognized by the peptidase. We shall see below that ER signal peptides that are contained within the polypeptide chain rather than at the amino terminus do not have these recognition sites and are never cleaved; instead, they can serve to retain transmembrane proteins in the lipid bilayer after the translocation process has been completed.

The amino-terminal ER signal peptide of a soluble protein itself has two signaling functions: in addition to directing the protein to the ER membrane, it is thought to serve as a **start-transfer signal,** which remains bound to the translocation apparatus while the rest of the protein is threaded continuously through the membrane as a large loop. Once the carboxyl terminus of the protein has passed through the membrane, the signal peptide is released from the translocator pore, cleaved off by the signal peptidase, and rapidly degraded to amino acids by other proteases in the ER while the protein is released into the ER lumen (Figure 12–43).

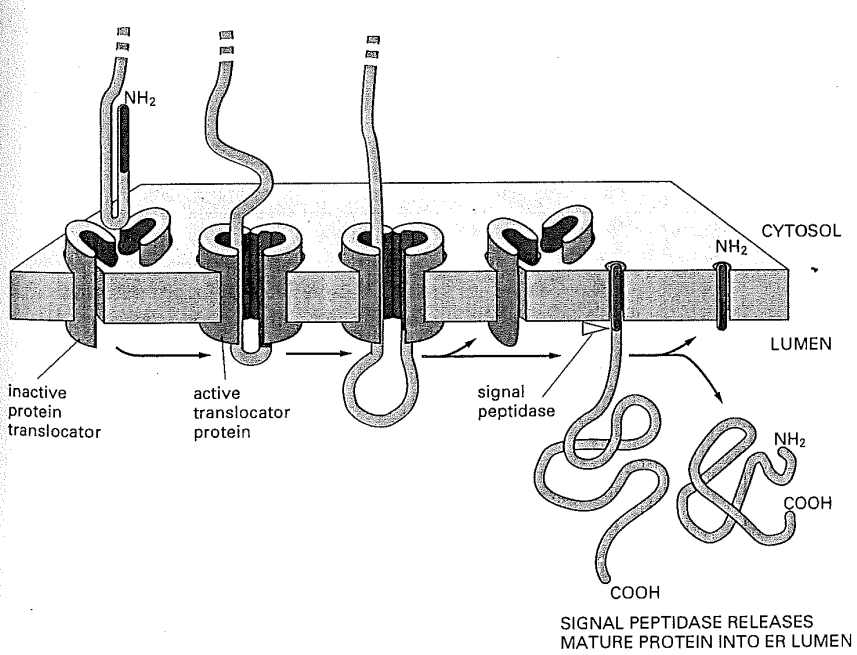


Figure 12–43 **The translocation of a soluble protein across the ER membrane.** In this hypothetical model the protein translocator in the membrane is postulated to exist in two alternative states—active or inactive. On binding an ER signal peptide (which acts as a start-transfer signal), the translocator adopts an active state and begins to transfer the polypeptide chain across the lipid bilayer as a loop. In this state it forms an aqueous pore across the membrane that can be detected electrophysiologically if the polypeptide chain is released (see Figure 12–42). After the protein has been completely translocated, the translocator reverts to an inactive conformation, which can no longer conduct ions across the membrane but is open to the lipid bilayer, allowing the hydrophobic signal peptide to diffuse out into the bilayer, where it is rapidly degraded. In this and the following two figures the ribosomes have been omitted for clarity.

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Europäisches Patentamt
European Patent Office
Office européen des brevets

Letter accompanying subsequently filed items

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1	Reply to examination report	Letter to EPO Response to Summons N411599EP-B PXT.pdf	EXRE3-1.pdf
2	Reply to examination report	Second Andrien Declaration signed 15 Aug 18 N411599EP-B.PDF	EXRE3-2.PDF
3	Reply to examination report	Alberts et al (1994) signal peptide extract.PDF	EXRE3-3.PDF
4	Reply to examination report	Opposition Statement against EP1720571 (D11) N411599EP-B.PDF	EXRE3-4.PDF
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Date:	**15 August 2018**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

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.
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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 Croskery M.D October 4, 2002
Alexion Clinical Director Date

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[redacted]

[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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[redacted]

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[redacted]

6.6 Drug Accountability

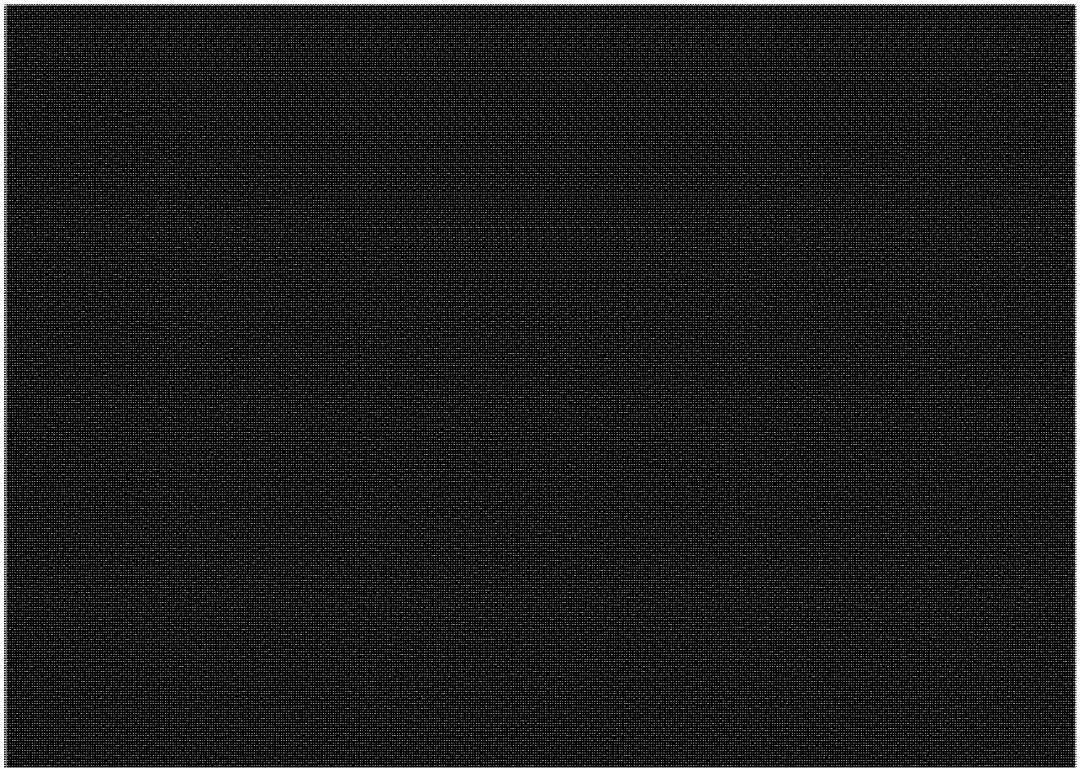
When a drug shipment is received at the site, the pharmacist at each site should verify the contents and sign the Drug Order Form provided with the shipment and maintain the original copy for collection by the Study Monitor/Research Associate. The duplicate copy should be kept in the Pharmacy Binder.

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]

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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.

SPONSOR SIGNATURE PAGE

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

PROTOCOL NUMBER: C04-001

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

3-June-04
Date

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

3-June-04
Date

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4.5. Investigational Product Packaging and Storage

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

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

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

4.6. Test Article Accountability

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

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

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

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

[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

SECOND DECLARATION BY DR BRUCE ANDRIEN

I, Bruce Andrien, Director, Early Assay Development and Pharmaceutical Development, of Alexion Pharmaceuticals, Inc., of 100 College Street, New Haven, CT 06510, United States of America, make the following factual declaration concerning the light chain sequence of eculizumab:

1. I am Director, Early Assay Development and Pharmaceutical Development, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which was attached with my declaration of 23 May 2017.

2. Through my work at Alexion over the past 17 years, I have become very familiar with the protein sequences and structures of antibodies.

3. I understand that EP 16195355.9 is a European patent application whose parent international application was filed on March 16, 2007.

4. I understand the pending claim 1 of EP 16195355.9 reads as follows:

> *"An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4."*

5. I understand that SEQ ID NO: 4 in EP 16195355.9 reads as follows:

> SEQ ID NO: 4 - Eculizumab Light chain
>
> MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
> NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
> NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASWCLLNNFYPREAKV
> QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
> LSSPVTKSFNRGEC

6. I understand that EP 16195355.9 also refers to an antibody comprising a light chain variable region that consists of SEQ ID NO: 3.

> SEQ ID NO: 3 - Eculizumab V_L
>
> MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
> NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
> NVLNTPLTFGQGTKVEIKRT

7. I have been asked to comment on whether one skilled in the art of antibody structure would have immediately recognized that the Applicant of EP 16195355.9 did not intend to seek protection for an antibody comprising the whole of SEQ ID NO: 4, or the whole of SEQ ID NO: 3.

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8. In my Declaration of 23 May 2017, I explained why one skilled in the art in 2007 would have immediately recognized that the signal peptide is included in SEQ ID NO: 4 and would have found it straightforward and simple to delineate the signal peptide from the active N-terminus of the light chain in SEQ ID NO: 4. The same applies to the signal peptide and active N-terminus of the light chain variable region in SEQ ID NO: 3.

9. One skilled in the art would have also known in 2007 that the signal peptide portion of SEQ ID NO: 4, or SEQ ID NO: 3, would not be present in the mature antibody.

10. As described, for example, in Alberts et al. (1994) (attached as Exhibit A), secretory proteins are initially produced as precursor polypeptides. Each precursor polypeptide contains a signal peptide that directs the polypeptide into the endoplasmic reticulum for further transport and secretion. Where the signal peptide is present at the N-terminus of the polypeptide, it is cleaved off after being transported into the endoplasmic reticulum.

11. It was known in 2007 that an antibody heavy or light chain protein is initially produced as a precursor polypeptide containing a signal peptide, as explained in my Declaration of 23 May 2017. It was also well known that, as for other secretory proteins, the signal peptide of the antibody heavy or light chain directs the polypeptide into the endoplasmic reticulum and is cleaved off during transport of the antibody heavy or light chain into the endoplasmic reticulum. As a result of this cleavage, the mature antibody comprising a heavy chain and a light chain never comprises a signal peptide in either chain.

12. Thus, one skilled in the art would immediately have recognized that the Applicant of EP 16195355.9 intended to obtain protection for an antibody that contains only the mature portion of the light chain shown in SEQ ID NO: 4 (i.e., residues 23 to 214 of SEQ ID NO: 4), or SEQ ID NO: 3 (i.e., residues 23 to 109 of SEQ ID NO: 3), and not for an antibody that contains the whole of SEQ ID NO: 4, or SEQ ID NO: 3.

DECLARED at New Haven, CT
United States of America

This 15th Day of August 2018

Bruce A. Andrien Jr.

Bruce A. Andrien Jr., Ph.D.

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

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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).

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There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 32, lines 7-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 32, lines 12-14) is not specified in the claim, despite being an essential feature of the Example. Finally, the breakthrough occurred in the extension study between weeks 12 and 64 (page 32 lines 17-20; page 35, lines 8-9) whereas the claim is not so limited and covers treatment regimens where the shorter 12 day treatment cycle is initiated in the initial 12 week acute phase of treatment.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the description or claims of the application as filed and are not allowable according to Art.123(2) and Art.100(c) EPC. The dependent claims, which all contain the features of claims 1 and 5, also contravene these provisions.

5- Lack of novelty (Article 100(a)/Art 54 EPC)

5.1 – The subject matter of claim 1 and 5 is not entitled to the priority

According to Art.87(1) EPC, the priority of an earlier application may be claimed if it discloses "the same invention". According to the Guidelines for Examination (F-VI, 1.3) and G2/98, this is to be interpreted to mean that the subject matter of a claim in a European patent may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole.

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed in the priority application (US patent application No. 10/771,552 filed on 03 February 2004), in contravention of Art.87(1). The claims are therefore not entitled to the priority date.

Claims 1 and 5 of the opposed patent contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the priority application of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any

features relating to the route of administration or dosage regimen. Therefore, for the priority claim to be valid, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description of the priority application discloses feature (2) on page 4, lines 13-14: "Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as hemoglobinuria, anemia, fatigue, low red blood cell count, erectile dysfunction, dysphagia and thrombosis (page 22, lines 12-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 7-9). From this broad class, anti-C5 antibodies are described as particularly useful (page 11, lines 9-11) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 17, line 23 – page 18, line 8).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 19, lines 15-18). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description in the priority application of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgement of the prescribing physician (page 19, lines 12-14). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the invention presented on pages 30-35. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (no route of administration specified) and then with 900 mg eculizumab on a bi-weekly basis (page 30, lines 6-16; no route of administration specified). One of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 32, lines 13-19).

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

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(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

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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.

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- 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

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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."*

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Further, in Reasons 7.3.2 the Board stated that:

> *"The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step."*

This decision is applicable to the present situation - the use of the defined dosage schedule would have been considered routine to the skilled person in view of the prior art and faced with the technical problem.

Therefore claim 1 lack inventive step over D5 alone or in combination with any one of D2, D3, D4 or D6, or over any of D2, D3, D4 or D6. Claim 5 which relates to the same features also lack inventive step for the same reasons.

The additional features of dependant claims 2 to 4 and 5 to 8 do not confer inventive step the claimed use.

7- Insufficiency of disclosure (Art 100(b)/Art 83 EPC)

7.1 Claims 1 to 8 contravene Article 83 because they lack essential features for the alleged technical effect

Eculizumab is an essential feature of claims 1 to 8. The structure of Eculizumab is not disclosed in the application as filed. On page 8, lines 8-9, the patent discloses that h5G1.1-mAb is currently undergoing clinical trials under the tradename Eculizumab. On the same page, lines 5 to 8, the patent further mentions that methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 (D9) and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996 (D10).

However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.

D9 is a patent assigned to Alexion Pharmaceutical, Inc which relates to C-5 specific antibodies for the treatment of inflammatory diseases.

In example 7, D9 discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9 lists a number of humanized, chimeric and non humanized scFv, Fd and light chains. D9 does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.

D10 is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10 discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1390), the humanization of 5G1.1 variable regions and the construction and expression of various Fab and scFv (see page 1391). The sequences of humanized variable regions are provided in Figure 1. The construction of a humanized antibody h5G1.1 called

h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col.1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10.

In view of the above, none of the patent, D9 or D10 provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.

Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8.

Therefore claims 1 to 8 are not sufficiently disclosed in the sense of Article 83 EPC.

7.2 Claims 1 to 8 contravene Article 83 because the invention is not disclosed in a manner sufficiently clear and complete.

Claim 1 as granted comprises the feature that the "patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)".

The term "breakthrough in serum hemolytic activity" is neither defined in claim 1 nor in the general disclosure of the application as filed. This term is used in the example section (see page 35, lines 9-10) and in particular in connection with two patients who did not sustain levels of Eculizumab necessary to consistently block complement. Again, no definition of this term is provided in the example. On page 35, lines 11 to 15, the application as filed discloses that "breakthrough of complement blockade resulted in hemoglobuniria, dysphagia and increased LDH and AST which correlated with the return of the hemolytic activity". These events (hemoglobinuria, dysphagia and increased LDH and AST) seem to be associated to some extent with serum hemolytic activity. However, it is not clear if one, two, three or four of these events are necessary and/or sufficient to characterize a breakthrough according to claim 1.

Claim 1 does not provide any indication relative to the characterization of "breakthrough in serum hemolytic activity". In the absence of additional guidance from the description, the skilled person would not be able to determine when a patient exhibits breakthrough in serum hemolytic activity and as a consequence would not be able to determine if and when the dosing interval should be reduced to 12 days.

Therefore, the skilled person would not be able to carry out the subject matter of claims 1 to 8 which therefore contravene Art.83 EPC.

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	6620529
Application number	EP16195355.9
Date of receipt	15 August 2018
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file

Documents submitted	
package-data.xml	ep-sfd-request.xml
epf1038.pdf (1 p.)	EXRE3-1.pdf\Letter to EPO Response to Summons N411599EP-B PXT.pdf (14 p.)
EXRE3-2.PDF\Second Andrien Declaration signed 15 Aug 18 N411599EP-B.PDF (2 p.)	EXRE3-3.PDF\Alberts et al (1994) signal peptide extract.PDF (3 p.)
EXRE3-4.PDF\Opposition Statement against EP1720571 (D11) N411599EP-B.PDF (13 p.)	EXRE3-5.PDF\C04-001 REDACTED N411599EP-B PXT.PDF (9 p.)
EXRE3-6.PDF\C02-002 REDACTED2 N411599EP-B PXT.PDF (6 p.)	

Submitted by	CN=Pamela Tuxworth 23470
Method of submission	Online
Date and time receipt generated	15 August 2018, 17:44 (CEST)
Message Digest	EC:B9:D1:84:6F:BB:37:B7:F4:70:C1:64:AF:F2:72:0F:CC:F8:48:F0

Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Application No. : 16 195 355.9

Consultation by telephone with the applicant / representative

Despatch for information

Participants

Applicant: Alexion Pharmaceuticals, Inc.

Representative: J A Kemp - Pamela Tuxworth

Member(s) of the Examining Division: Collura, Alessandra

Result of consultation

See Separate Sheet

14.09.2018
. . . . . .
Date

Collura, Alessandra
. . . . . .
Examiner

Enclosure(s):

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Application No.	Ref.	Date
16 195 355.9 - 1112		25.09.2018
Applicant Alexion Pharmaceuticals, Inc.		

Result of consultation

A copy of the result of consultation of 14.09.2018 is enclosed for your information.

Collura, Alessandra
For the Examining Division

Enclosure(s): Copy of result of consultation (Form 2036)

The representative of the applicant wanted to discuss the application with the first examiner.

The first examiner explained that, since the application related to some core legal issues, the examining division would probably have to be enlarged with a legal member. The first examiner will phone again the representative of the applicant once the situation of the application has been clarified (probably within next week).

The first examiner confirmed the maintenance of the oral proceedings.

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Application No. : 16 195 355.9

Consultation by telephone with the applicant / representative

Despatch for information

Participants

Applicant: Alexion Pharmaceuticals, Inc.

Representative: Pamela Tuxworth

Member(s) of the Examining Division: Collura, Alessandra

Result of consultation

See Separate Sheet

19.12.2018
. . . . . .
Date

Collura, Alessandra
. . . . . .
Examiner

Enclosure(s):

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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Brell, Eva

J A Kemp
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ROYAUME UNI

Date
02.01.2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Oral Proceedings on 05.02.19 at 09:00 hours

Subject:
- ☐ Your letter of .....
- ☒ Cancellation / postponement at the instigation of the division

Communication:

1. ☐ The date / time fixed for oral proceedings is maintained.
   - ☐ The reasons are indicated on enclosed EPO Form 2906.
2. ☐ The request for the oral proceedings to be held as a videoconference is rejected. The reasons are indicated on enclosed EPO Form 2906.
3. ☐ The above-mentioned oral proceedings will start at ..... hours.
4. ☒ The summons to attend oral proceedings on the above-mentioned date is cancelled.

4.1
- ☐ The reasons are indicated on enclosed EPO Form 2906.
- ☐ The procedure will be continued in writing.
- ☒ A new date will be set later.
- ☐ New summons will follow.

4.2 ☐ Due to administrative reasons the oral proceedings have to be postponed to a later date. New summons will follow.

4.3 ☐ The application is deemed to be withdrawn. The right to oral proceedings only persists as long as proceedings are pending.

Please take note.

Please confirm receipt of this communication by return with date stamp and signature to our central fax server (+49) 89 23 99 44 65.

Registered letter

Samsung Bioepis Exhibit No. 1028
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For the Examining Division

Enclosure : EPO Form 2906

Samsung Bioepis Exhibit No. 1028
Page 331

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Substantive Examiner
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Tel: +49 89 2399 - 7870

Application No.	Ref.	Date
16 195 355.9 - 1112		02.01.2019
Applicant Alexion Pharmaceuticals, Inc.		

Result of consultation

A copy of the result of consultation of 19.12.2018 is enclosed for your information.

Collura, Alessandra
For the Examining Division

Enclosure(s): Copy of result of consultation (Form 2036)

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Application No. : 16 195 355.9

Consultation by telephone with the applicant / representative

Despatch for information

Participants

Applicant:	Alexion Pharmaceuticals, Inc.
Representative:	Pamela Tuxworth
Member(s) of the Examining Division:	Collura, Alessandra

Result of consultation

See Separate Sheet

19.12.2018
. . . . . .
Date

Collura, Alessandra
. . . . . .
Examiner

Enclosure(s):

The first examiner informed the representative of the applicant of the fact that the summons to oral proceedings on 05.02.2019 should be cancelled because the legal member who was assigned to the file could not attend the oral proceedings.

A new date for the oral proceedings will be set at the beginning of January 2019.

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Application No.	Ref.	Date
16 195 355.9 - 1112		02.01.2019
Applicant Alexion Pharmaceuticals, Inc.		

Result of consultation

A copy of the result of consultation of 19.12.2018 is enclosed for your information.

Collura, Alessandra
For the Examining Division

Enclosure(s): Copy of result of consultation (Form 2036)

The first examiner informed the representative of the applicant of the fact that the summons to oral proceedings on 05.02.2019 should be cancelled because the legal member who was assigned to the file could not attend the oral proceedings.

A new date for the oral proceedings will be set at the beginning of January 2019.

Samsung Bioepis Exhibit No. 1028
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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Stark, Saskia

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ROYAUME UNI

Date
27.03.2019

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 05.09.19 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 05.07.19.

The actual room number as well as the waiting room numbers will be given to you by the porter in the foyer at the above EPO address.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at one of the porters' lodges in the PschorrHöfe, the parking ticket will be revoked.

Please note that the oral proceedings will always take place as scheduled unless you are otherwise informed. The EPO will generally not send a reminder or a further communication prior to the date of oral proceedings.

1st Examiner:	2nd Examiner:	Chairman:	Legally qualified member:
Collura, Alessandra	Rodríguez-Palmero, M	Orlando, Michele	Kley, Britta

Registered letter with advice of delivery
EPO Form 2008 09.18 [ORAL03=9999] (20/03/19)

to EPO postal service: 20.03.19

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For the Examining Division

Annexes:
Confirmation of receipt (Form 2936)
Communication (EPO Form 2906)

Samsung Bioepis Exhibit No. 1028
Page 338

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.

The applicant should also be aware of documents D8 and D9 filed by Third Parties on 12.12.2017 as well as of WO2005/074607 (D10) which is the publication pamphlet of EP1720571, referred to in D9. All these documents as well as the submissions of the applicant, especially the brief submitted on 15 August 2018, may form the basis of the discussion during the oral proceedings.

Moreover, a further document (D11), which has been cited in the present application (see page 16, lines 19-24) is referred to in the summons and will be discussed at the oral proceedings. A copy of the document will be annexed to the present communication.

D1: US2005/191298

D2: Hillmen et al. (2003)

D3: Hillmen et al. (2004)

D4: Harding (2004)

D5: Hill (2005)

D6: Database Registry

D7: Thomas et al. (1996)

D8: Table of Alexion European patents and patent applications on eculizumab

D9: Submission of Alexion dated December 30, 2013 in opposition proceedings against EP1720571

D10: WO2005/074607

D11: US6355245

The documents attached to the letters of the applicant to the examining division will also be addressed at the oral proceedings if necessary.

Due to the underlying discussion relating to the disclosure of the sequence for *eculizumab* the examining division has been enlarged under Article 18(2) EPC by a legally qualified member on 1 October 2018. The so enlarged examining division has decided to postpone the oral proceedings initially set for 5 February 2019 and to combine the present proceedings for reasons of procedural economy with the proceedings in the parallel case relating to EP 16 172 335.8 for the purposes of the oral proceedings. Both applications are divisional applications from EP 11 001 632.6, both examination proceedings are conducted by the same representatives for the same applicant before identically staffed examining divisions. In both proceedings the same fundamental problem arises which is decisive for both proceedings, namely the extent to which the prior art disclosing eculizumab has an impact on the patentability of the two applications pending before the examining division. After discussion of this fundamental issue, the proceedings will be dealt with successively, if necessary, during the same oral proceedings on the same day.

Regarding the present application EP 16 195 355.9 the enlarged examining division refers to the annex to the summons issued on 7 May 2018. It upholds its preliminary opinion which is amended as follows:

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.

With the amendments filed on 16.01.2018, the applicant amended page 5 of the description of the application (which is the same as the description of the parent application), and introduced a limitation to the length of the sequence of the indicated SEQ ID NO:3 and SEQ ID NO:4.

The applicant also amended the claims and introduced the same limitation to the length of the SEQ ID NO:4 in present claim 1.

In particular, instead of the full length of the sequences 3 and 4 provided in the application, the specific embodiments disclosed on page 5 and claimed in claim 1 are presently directed to:

(i) an antibody having a heavy chain variable region consisting of SEQ ID NO:1 and a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3; and

(ii) an antibody (as claimed) comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4.

In his letter dated 16.01.2018, the applicant explained that the amendments that he had carried out were to be considered the correction of an obvious mistake which met the requirements of Rule 139 EPC.

As already stated in the official communication from the examining division dated 17.11.17, it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and in the application as originally filed were actually those for which protection was sought.

Moreover, neither in the original nor in the parent application a disclosure was present of the fact that sequences 3 and 4 did not have to include the leader peptide.

For the reasons given above, the examining division concludes that the subject-matter of the present application does not meet the requirements of Articles 76(1) and 123(2) EPC.

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 (see D9 relating to the opposition to EP1720571 filing date 03.02.2005; priority date 03.02.2004, in which the applicant was the patentee) had stated that the sequence for eculizumab had been submitted to Chemical Abstracts Services (CAS) and entered STN database on 14.02.1999 (see also the document D6 cited in the European Search Report).

In particular the applicant, at the time of the opposition proceedings against EP1720571, had argued against the objection of lack of sufficiency of disclosure raised by the opponent and based on the allegation that the complete structure of eculizumab was neither disclosed in the application as filed nor in any of the prior art documents cited by the opponent.

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 argued that the sequence was not correct.

In support of his arguments, the applicant provided the correspondence between Mr Saxe, who was the primary intellectual property counsel at Alexion Pharmaceuticals, and Mr Bishop, from the CAS Registry database. From this correspondence, it can be seen that Alexion had filed a sequence of eculizumab which contained some mistakes and that the corrected sequence was substituted to the wrong one in May 2009.

The applicant also:

(i) provided evidence of the fact that the marketing approval from the FDA for eculizumab was received by Alexion on 16.03.2007, i.e. after the filing date of the present application;

(ii) stated that when eculizumab was made available for clinical trials, all physicians and participants were required to maintain Alexion's confidential and/or proprietary information in strict confidence. For that reason the skilled person couldn't have obtained and analysed eculizumab to determine its structure; and

(iii) affirmed that the third party observation filed on 12.12.2017 were merely circumstantial and did not provide evidence that eculizumab was publically available at the date of filing of the application.

The examining division considered all the arguments provided in respect to the disputed question of whether eculizumab was publically available or not at the filing date of the present application.

The opinion of the examining division is the following:

1. The fact that Alexion has received the marketing authorisation for eculizumab from the FDA on 16.03.2007 does not necessarily mean that the antibody was not available to the public or that is structure was not known before the filing/priority of the present application.

2. It is clear from Mr. Saxe's declaration that Alexion Pharmaceuticals had intended to submit the correct sequence of eculizumab to CAS and that the incorrect sequence was submitted only inadvertently.

This means that (i) there was no interest in keeping the substance secret after the date of submission to CAS and (ii) anybody to whom the correction of the mistake in the sequence would not have been obvious could have contacted Alexion Pharmaceuticals Inc. to obtain the correct sequence.

Even if it were now purported that Alexion Pharmaceuticals would have never disclosed the correct sequence to anybody who had asked them for it, that would not amount to a valid defence since under the legal principle of "*nemo potest venire contra factum proprium*" Alexion Pharmaceuticals would be bound to its declared intention to lay the compound open under the CAS rules in order to have other patents be regarded as sufficiently disclosed.

The statement from the applicant acting as patentee in EP1720571 (see D9, page 14, item 5.1.2) confirms the fact that he considered the structure of eculizumab to have been disclosed at the filing date of the present application. To be noted is also the fact that the date of the applicant's statement in D9, namely the 30.12.2013, is later than the date in which the correction to the eculizumab structure was requested to CAS.

3. The many patent and non-patent literature documents published before the priority date and relating to the use of eculizumab can be regarded as evidence that the compound was well-known and available to the public before the priority date of the patent application. Among these documents, different published patent applications belonging to the applicant can be seen (see for example D8 filed by Third Party on 12.12.2017 and in particular WO2005/074607 published on 18.08.2005, which is the international publication pamphlet of EP1720571).

4. In respect to the statement of the applicant that the physicians and participants to the studies referred to in the prior art were bound to an agreement of secrecy and confidentiality, a proof of the confidentiality agreement between the company and each participant/physician involved in the studies should be provided.

Moreover, it has to be noted by the applicant that none of the documents cited in respect of the present application relate to clinical trials and therefore the list of the confidentiality agreements filed by the applicant with the declaration of Laural Boone (see letter dated 10.07.2017) does not apply to the present case.

For the reasons given above, the examining division concludes that the structure of eculizumab was available to the public at the filing/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 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.

2.4 The present application lacks novelty over the disclosure of D11

The description of the invention (see page 16, lines 19- 24) refers to D11 and states that the document discloses the preferred whole antibody named eculizumab. That would mean that the eculizumab claimed in the patent and the one disclosed in D11 is the same antibody.

3. Article 56 EPC

Should the applicant be able to prove to the examining division that the amendments carried out on the application in respect to the sequence of eculizumab represented the correction of an obvious mistake and should he succeed with the argument that the full correct sequence of eculizumab was not disclosed to the public prior to 16 March 2007 and the present application was assumed to be novel, it would still not be inventive compared to the available documents of the prior art disclosing eculizumab (see D1-D5 and D10, D11).

The difference between D1-D5 and D10, D11 and the present invention would lie in the sequence of the claimed compound. Apart from that, the present patent application and the the cited documents of the prior art are directed to the same purpose.

In this respect the applicant contends – and provides evidence – that "a sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999" under the CAS entry No. 219685-50-4. At the same time the applicant points out that the submission to CAS of 14 February 1999 was incorrect, even though it was made in good faith. It was not until 20 April 2009 that the applicant learnt of these errors in the sequence listing and requested correction of CAS Registry Number 219685-50-4 by providing complete and accurate amino acid sequences for eculizumab.

In detail, the initially submitted sequence contained a heavy chain which was longer the the actual one (i.e. 650 amino acids vs 448 amino acids) and the light chain contained one single error in the sequence, i.e. the inclusion of an arginine (R) residue at position 38 of the light chain sequence instead of a glutamine (Q).

It follows from this contention that the technical problem to be solved by the present application is the provision of the corrected sequence known to be associated to the name eculizumab in the cited prior art documents D1-D5 and D10, D11. However, the correction of an error in a registered sequence listing can hardly qualify as progress of technology or contribution to the art that does not already follow logically from the closest prior art. Rather, it remains to be a correction without own inventive

character. As a matter of fact, at the filing date of the present application the skilled person had the knowledge to carry out the sequence of eculizumab (see teaching of D7).

The now not improved but simply corrected sequence is the contribution to technology that had already been available to the public for long before the filing date of the present application. Without additional technological process, there can be no finding of inventive step (see Guidelines Chapter VII-4) and eventually no monopoly right.

4. Article 83 EPC

The attention of the applicant is drawn to the fact that in case 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.

Samsung Bioepis Exhibit No. 1028
Page 347

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Date
27.03.2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2008

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Acknowledgement	Signed Acknowledgement N411599EP-B PXT.PDF	ADVOFDELIVRY-1.PDF

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Oxford, GB**
Date:	**05 April 2019**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

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Date
27.03.2019

Reference	Application No./Patent No.
N4115996P-B	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2008

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	7356072
Application number	EP16195355.9
Date of receipt	05 April 2019
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file
Documents submitted	package-data.xml epf1038.pdf (1 p.) ep-sfd-request.xml ADVOFDELIVRY-1.PDF\Signed Acknowledgement N411599EP-B PXT.PDF (1 p.)
Submitted by	CN=Pamela Tuxworth 23470
Method of submission	Online
Date and time receipt generated	05 April 2019, 12:09 (CEST)
Message Digest	B0:91:D7:C6:FE:83:35:87:0C:3D:33:5D:37:04:E4:8D:FF:3D:6E:39

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

5 July 2019

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

Thank you for the summons to attend oral proceedings dated 27 March 2019. The Applicant wishes to make the following submissions in advance of the hearing scheduled for 5 September 2019.

The claims currently on file form the basis of our main request.

Three auxiliary requests are attached and we ask that the Examining Division consider the auxiliary requests in the event that the main request is considered unallowable.

Tracked-changes versions of the main request showing the amendments being made to derive the first two auxiliary requests and of the first auxiliary request showing the amendments being made to derive the third auxiliary request are attached.

I attach a full list of the documents cited in these examination proceedings. A copy of each of D24 to D31 is attached.

The present claims

Claim 1 of the present application is directed to:

> "*An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.*"

Claim 2 is directed to a pharmaceutical composition comprising this antibody.

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The present application discloses that SEQ ID NO: 2 is the full (variable and constant) heavy chain amino acid sequence of eculizumab, and that SEQ ID NO: 4 is the full (variable and constant) light chain amino acid sequence of eculizumab. This is confirmed in paragraph 6 of the Boone declaration (D13). SEQ ID NO: 2 is the mature sequence and does not include the N-terminal signal peptide. The heavy chain constant region of SEQ ID NO: 2 is a unique, engineered heavy chain constant region. SEQ ID NO: 4 is an immature sequence that includes the 22 amino acid N-terminal signal peptide, which is cleaved off during antibody production in a cell. The light chain constant region of SEQ ID NO: 4 is a kappa constant region.

The claims are therefore directed to the specific antibody, eculizumab, which obtained marketing approval under the trade name SOLIRIS® after the filing date of the present application (see D15/D16).

The structure of eculizumab was not disclosed in the prior art

The full heavy chain and light chain amino acid sequences of SOLIRIS® (eculizumab) were first disclosed in the present application. Prior to the filing date of the present application, it was not known that "eculizumab" was an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

D1-D5 do not disclose the structure of the claimed antibody

None of D1-D5 includes any structural information for "eculizumab".

D1 refers to "eculizumab" as being the tradename of the antibody "*h5G1.1-mAb*". D1 does not include any sequence information, but states that:

> "*Methods for the preparation of h5G1.1-mAb, h5Gl.l-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.*" (D1, page 5, left column last two lines to right column line 8).

U.S. Pat. No. 6,355,245 is D11, and Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996 is D7 in the present proceedings.

D2 describes "eculizumab" as "*a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). No structural information is provided in D2, and it does not reference any other documents.

D3 describes "eculizumab" as "*a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components*[14,15]" (D3, left column page 553, third paragraph, first three lines). Reference 14 in D3 is a general publication relating to humanization of antibodies (Riechmann et al. (1988) "Reshaping human antibodies for therapy" Nature 332: 323-7), and reference 15 is D7 in the present proceedings. No structural information is provided in D3 itself.

D4 describes "eculizumab" as "*a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12)*" (D4, page 673, right column, third paragraph, first three lines). Reference 12 in D4 is D7 in the present proceedings. D4 also includes the CAS STN database number "CAS: 219685-50-4" on page 673. However, as discussed below, this database did not include the correct sequence for eculizumab at the filing date of the present application. No structural information is provided in D4 itself.

D5 defines "eculizumab" as "*a monoclonal antibody that blocks the complement system at C5"* (D5, page 849, left column, last two lines of answer to first question). No structural information for "eculizumab" is provided in D5.

Thus, D1 to D5 do not provide any sequences for "eculizumab", and only point to D7 and/or D11 as describing "eculizumab".

Neither D7 nor D11 discloses the full structure of the claimed antibody

D11 and D7 are referred to in the cited prior art documents as describing "eculizumab". However, neither D7 nor D11 discloses the amino acid sequences of SOLIRIS® (eculizumab) recited in the present claims.

D7 and D11 describe the cloning of the murine 5G1.1 antibody and the generation of humanised variable region sequences.

The only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

D7 discloses CDR grafted V_L and V_H sequences (h5G1.1VCH and h5G1.1VLC in Figure 1 of D7) and also V_L and V_H sequences comprising back mutations (h5G1.1VHC+F and h5G1.1VLC+F in Figure 1 of D7) and reports that an intact humanised antibody of the IgG4 isotype was constructed and produced (see page 1396, paragraph spanning columns of D7).

As discussed above, the present claims are directed to an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4, which are the sequences of the antibody in SOLIRIS® (eculizumab). This claimed antibody is **not** the IgG4 antibody described in D7. In particular, eculizumab does not have an IgG4 heavy chain constant region, but a unique, engineered, non-naturally occurring constant region sequence that has 14 different amino acid residues compared to the IgG4 sequence (4 in the CH1 domain, 6 in the hinge region and 4 in the CH2 domain - see paragraphs 4 and 5 of the declaration of Dr Leonard Bell (D12)).

Therefore, although D1 refers to D7 and D11, and D3 and D4 refer to D7, supposedly as describing "eculizumab", neither D7 nor D11 discloses the full heavy chain sequence shown in SEQ ID NO: 2 of the present application and the full light chain sequence shown in SEQ ID NO: 4 of the present application.

With regard to D11, the Examining Division has argued that:

> "*The description of the invention (see page 16, lines 19-24) refers to D11 and states that the document discloses the preferred whole antibody named eculizumab. That would mean that the eculizumab claimed in the patent and the one disclosed in D11 is the same antibody*" (page 7, first paragraph of the summons dated 23 March 2019).

Page 16, lines 24-25 of the present application states that: "*These antibodies disclosed in U.S. Patent 6,355,245 [D11] include a preferred whole antibody (now named eculizumab)*". However, it is necessary to look at D11 to see what it actually discloses, and despite the statement made in the present application, D11 does not in fact disclose the sequences of any whole antibody.

As discussed above, the only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

The statement in the present application is therefore correctly interpreted as meaning that D11 discloses a genus of antibodies that includes the preferred antibody of the present invention (eculizumab), and not that this antibody is explicitly disclosed in D11. It is a matter of fact that D11 does not disclose an antibody with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 as presently claimed.

None of D1-D5, D7 and D11 provide an enabling disclosure of the claimed antibody

In view of the above, the skilled person would not be able to reproduce "eculizumab" based on the disclosures of any of D1-D5, D7 and D11. According to the established case law, a disclosure in a prior art document is novelty-destroying only if the teaching it contains is reproducible (see, for example, T 1437/07, Reason 25, and Guidelines for Examination G-IV-2).

Hence, none of D1-D5 can be considered to belong to the state of the art according to Article 54(2) EPC, at least in as far as they relate to "eculizumab", and consequently none of D1-D5 prejudices the patentability of the claimed invention.

None of the patents listed in D8 provide an enabling disclosure of the claimed antibody

D8 lists a number of Alexion patents and patent applications that refer to "eculizumab". None of the patents and patent applications listed in D8 provide any sequence information for "eculizumab". Insofar as these patents and patent

applications use the term "eculizumab", they refer back to D7 and/or D11 as shown in the attached table (D24).

As discussed above, neither D7 nor D11 disclose an anti-C5 antibody as presently claimed, with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4. These full sequences of the antibody in SOLIRIS® (eculizumab) were not known in the art at the filing date of the present application. Therefore, the prior art disclosures in the patents and patent applications do not enable the skilled person to make the specific antibody that is now claimed.

In this regard, it should be noted that the patent and patent applications listed in D8 are not focused only on eculizumab. These patents and applications relate to complement inhibitors more broadly, including anti-C5 antibodies generally. As shown in D8, with one exception (EP 1720571, the patent granted from D10, which has been revoked (see below)), "eculizumab" is referred to only in dependent claims. Therefore, the fact that there is no enabling disclosure of the specific antibody "eculizumab" does not render all of these patents and patent applications insufficient across their whole scope. The insufficiency extends only to the specific dependent claims that mention "eculizumab."

Comments made by Alexion in D9 in the course of opposition proceedings regarding a different patent do not change what was in fact disclosed in the prior art

The Examining Division considers that D10 destroys the novelty of the present claims because the applicant stated in D9 that the structure of the antibody had been made available to the public on 14 February 1999. However, the statement made in 2013 by the applicant in the opposition proceedings against the patent derived from D10 does not change what was disclosed in D10 in 2005, and does not retroactively make the disclosure of D10, or any other prior art document, enabling in so far as it relates to "eculizumab".

The issue of sufficiency of disclosure of "eculizumab" was raised in the opposition proceedings against EP 1720571 (the patent derived from D10). The Opponent observed that:

> *"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)* [D11 in the present proceedings] *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)* [D7 in the present proceedings].
>
> ***However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.***

> *D9* [D11] *is a patent assigned to Alexion Pharmaceuticals, Inc. which relates to C-5 specific antibodies for the treatment of inflammatory diseases.*
>
> *In example 7, D9* [D11] *discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9* [D11] *lists a number of humanized, chimeric and non humanized ScFv, Fd and light chains. D9* [D11] *does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.*
>
> *D10* [D7] *is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10* [D7] *discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1380), 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 5G1.1 called h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col. 1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10* [D7].
>
> *In view of the above, none of the patent, D9* [D11] *or D10* [D7] *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."* (See section 7.1 of D21)

In defence of its patent, Alexion replied to this attack by asserting that the sequence for eculizumab was publicly available and stated (in D9) that:

> "A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4."*

The copy of CAS entry no. 219685-560-4 provided did not include any sequences, the identity of the sequences made available on 14 February 1999 was not investigated at that time, and this argument was not challenged.

In the preliminary opinion that accompanied the summons to oral proceedings dated 14 January 2014 (D25), the Opposition Division expressed the opinion that:

> "I*n the absence of evidence that* eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed" (paragraph 2.29 of D25).*

The position of the Opposition Division was not further challenged by Alexion or discussed at the oral proceedings (see Alexion letter of 17 March 2014 (D26), which does not include any arguments relating to sufficiency of disclosure), and EP1720571 was revoked for lack of inventive step (see the Decision of the Opposition Division dated 24 June 2014 (D27)).

The statement made in defence of EP1720571 should have no bearing on the current proceedings. It does not change the facts of what was disclosed prior to the filing date of the present application. Nor did Alexion gain any advantage from the statement made in connection with the opposition to that earlier patent.

In accordance with the opinion of the Opponent and Opposition Division in the opposition against EP1720571 that this patent does not provide a sufficient disclosure of "eculizumab", the application for EP1720571, D10, also does not provide an enabling disclosure of "eculizumab" and does not destroy the novelty of the present claims, which are directed to the unique, engineered antibody in SOLIRIS®.

The sequence of eculizumab was not available in the CAS database at the filing date

Heavy and light chain amino acid sequences were submitted to the CAS Registry in 1999 under the name "eculizumab", but, as evidenced by the Saxe Declaration, the sequences submitted to CAS at this time were **not** the sequences of the antibody (eculizumab) in SOLIRIS® to which the present claims are directed. The correct sequences for eculizumab were later submitted to replace the incorrect sequences in May 2009. These facts are not disputed.

However, one argument put forward by the Examining Division is that the availability of incorrect sequences for eculizumab on the CAS Registry database amounts to a public disclosure of the correct sequences for eculizumab because it was the intention of Alexion to make the sequence of eculizumab public at the time, such that Alexion would be obliged to provide the correct sequence of eculizumab to anybody who requested it. The Examining Division has referred to "*CAS rules*" as obliging Alexion to provide such information. However, the Examining Division has not provided evidence that any such rules existed, and Alexion is not aware of any. The fact therefore remains that the correct sequence of eculizumab was not made available in the CAS Registry prior to the filing date of the present application.

The statement by the applicant in D9 ("A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4.")* does not change the fact that the wrong sequence was included in the CAS database in February 1999 and the fact that this incorrect sequence was still the sequence listed for "eculizumab" in the CAS database at the filing date of the present application. The context of the statement in D9 is explained above. Rather than speculate as to the intent of Alexion in 1999, the proper inquiry should be what in fact was disclosed at the filing date of the present application and whether a person skilled in the art would have known or been able to determine the structure of eculizumab, as recited in the present claims.

The Boards of Appeal have considered the prior art effect of documents that contain mistakes. In particular, as stated in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, in T 412/91 the board held that:

> *"in principle, what constituted the disclosure of a prior art document was governed not merely by the words actually used in its disclosure, but also by*

> *what the publication revealed to the skilled person as a matter of technical reality. If a statement was plainly wrong, whether because of its inherent improbability or because other material showed that it was wrong, then - although published - it did not form part of the state of the art. Conversely, if the skilled person could not see the statement was wrong, then it did form part of the prior art.*"

Similarly, in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, it is noted that:

> "*In T 230/01 the board noted that a document normally forms part of the state of the art, even if its disclosure is deficient, unless it can unequivocally be proven that the disclosure of the document is not enabling, or that the literal disclosure of the document is manifestly erroneous and does not represent the intended technical reality. Such a non-enabling or erroneous disclosure should then not be considered part of the state of the art.*"

Applying this reasoning to the present case, either the skilled person would recognise that the sequences of eculizumab in the CAS database must be wrong, in which case the incorrect information is not part of the state of the art, or the sequences in the CAS database do not destroy the novelty of the present claims.

The Board in T 591/90 considered that it could be assumed that a skilled person would try to correct recognisable errors in a prior document if, using his general technical knowledge and consulting the reference literature, he could see at once that the information in question was not correct. (See Case Law of the Boards of Appeal, 8th edition, I-C-4.9.)

In the present case, the skilled person would be unable to correct the errors in the eculizumab sequences in the CAS database based on his common general knowledge and by consulting the literature.

The Saxe declaration (D17) explains that the heavy chain sequence submitted to CAS was much longer (650 amino acids) than the actual heavy chain sequence (448 amino acids). The annotated copy of the sequences originally submitted to CAS attached as D28 (obtained by searching for CAS Registry Number: 219685-50-4 on http://commonchemistry.org/ and annotated by hand) shows how extensive the errors are. In particular, the heavy chain sequence originally submitted to CAS contains three stretches of additional amino acids, has two single amino acid substitutions and is missing a stretch of 16 amino acids compared to the correct sequence. For comparison, an annotated copy of the corrected sequences for CAS Registry Number: 219685-50-4 is attached as D28a.

As discussed above, the skilled person would not have known that eculizumab has a uniquely-engineered heavy chain, and so the skilled person would not have known what the correct heavy chain constant region sequence of eculizumab should be.

Also, the light chain submitted to CAS in 1999 contains a single amino acid substitution compared to the correct sequence in the variable region (see D28 and D28a). The light chain variable region sequence shown in D7 is the same as the

incorrect sequence at this position, and so the skilled person would have had no way of knowing that there was an error in the light chain variable region sequence.

Accordingly, the errors in the eculizumab sequences are not errors that would have been recognisable, or at least correctable, by a person skilled in the art at the filing date.

It is worth noting how the EPO would have treated an earlier patent application in which the Applicant had erroneously included the incorrect sequence for eculizumab, such as that submitted to the CAS Registry in 1999. In such a situation, it would have been the intention of the Applicant to disclose the sequence of eculizumab to the public in the patent application. However, the Applicant would not have been allowed to correct the sequence merely because it was his intention to make the correct sequence public and because the correct sequence was necessary to claim eculizumab and meet the sufficiency requirements of Article 83 EPC. To the contrary, the sequence in such a patent application could later be corrected only if it was immediately obvious that the sequence contained an error and also what the correct sequence should be (Rule 139 EPC). In other words, the Applicant's intent would not have been taken into consideration. We refer in this regard to Board of Appeal decision T1111/02 in which there were errors in a sequence provided in the patent application that led to a finding of insufficiency because the incorrect sequences could not be corrected under Rule 88 EPC (now Rule 139 EPC). Accordingly, it is the disclosure itself – rather than the intent of the Applicant – that is controlling. The same should apply in the present case (regardless of what the intent of the Applicant was in 1999).

The fact remains that the CAS database did not contain SEQ ID NOs: 2 and 4 of the present application at the filing date, and the incorrect sequences for eculizumab submitted to CAS in 1999 do not destroy the novelty of the present claims.

Eculizumab itself was not publicly available at the priority date

Eculizumab used in the clinical trials was not available to the public

The clinical trials described in the prior art did not make the antibody eculizumab available to the public. The position taken by the Examining Division in this regard is inconsistent with established jurisprudence of the Boards of Appeal.

We are filing herewith a new Declaration of Michael Bombara (D31) who managed many of the clinical trials that Alexion conducted in the late 1990s and early 2000s, and whose responsibilities included ensuring that all of the necessary contacts between Alexion and the principle investigators and/or their institutions or study sites were in place (see paragraph 2 of D31). Bombara confirms that no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of the clinical studies (see paragraph 17 of D31).

The Bombara declaration summarises Alexion's clinical trial practice prior to the present filing date and explains the multiple layers of confidentiality that were built into each clinical study to ensure that the principal investigators and study

institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence (see paragraph 5 of D31).

Bombara refers to one specific example of the agreements in place for the pilot PNH study of 11 PNH patients referenced in D1 to D5 (the "C02-001" study) and provides evidence of the agreements in place for this study. The declaration confirms that the authors on each of D1-D3 and D5 were affiliated with Alexion or one of the two institutions bound by these agreements (see paragraph 15 of D31). Bombara also states that similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s (see paragraph 16 of D31).

The Bombara declaration additionally explains that the investigators and institutions agreed to adhere to strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material, and provides an example of a drug disposal form (see paragraphs 12 and 13 of D31).

As an initial point, the Examining Division is incorrect that "*none of the documents cited in respect of the present application relate to clinical trials*". All of D1 to D5 relate to clinical trials carried out under the control of Alexion:

- D2 and D3 describe a pilot study that was conducted in 11 PNH patients for 12 weeks. This study is the "C02-001" study as identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D5 describes an extension of the "C02-001" study during which the PNH patients were treated for 52 weeks. This extension study is the "E02-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D1 describes a further extension of the "C02-001" study during which the PNH patients were treated for two years. This extension study is the "X03-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D4, which discloses Alexion Pharmaceuticals, Inc. as its source (page 675, right column) is a review paper that describes a series of clinical trials that were conducted with eculizumab. All of these trials are studies identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31):
    - The study in 117 patients with idiopathic membranous nephropathy is "C99-004".
    - The study in 40 rheumatoid arthritis patients is "C97-001".
    - The study in 209 rheumatoid arthritis patients is "C99-001".
    - The study in 368 rheumatoid arthritis patients is "C001-004".
    - The two month trial in 10 patients with dermatomyositis is "C99-006".
    - The phase II trial in membranous nephritis is "C99-004".
    - The trial in systemic lupus erythematosus is "C97-002".
    - The pilot and extension studies in 11 PNH patients are "C02-001" and "E02-001".

The Boone declaration (D13) explains that all of these trials were carried out under confidentiality agreements and that the eculizumab drug supplies used in the studies were tightly controlled. We have also provided direct evidence of the relevant

provisions in the "C02-002" and "C04-001" studies, by way of example. As is clear from paragraphs 7 to 9 of D13 and paragraphs 5 and 16 of D31, all of the studies listed in the table included substantively identical confidentiality provisions. It is also stated in paragraph 2 of the Bell declaration (D12) and paragraph 3 of the Bombara declaration (D31) that eculizumab was used in clinical trials before 15 March 2007 only under strict control and conditions of confidentiality.

The Examining Division is asking that we provide proof of a confidentiality agreement between Alexion and each participant/physician involved in the clinical studies. This level of proof is not, however necessary.

It is stated in Case Law of the Boards of Appeal III-G-5.1.1 that:

> "*In ex parte proceedings the applicant bears the burden of proof for the facts in his favour, e.g. the fact that a document cited by the examining division does not form part of the state of the art (**T 160/92**, OJ 1995, 35)*",

However, this statement does not require that the Applicant provide absolute proof. The Case Law of the Boards of Appeal III-G-5.1.1 goes on, in the following paragraph to say:

> "*As far as issues relating to patentability requirements are concerned, the burden of proof in **examination proceedings** cannot lie initially with the applicant. If an examining division raises an objection, it must be appropriately substantiated.* In **T 578/06** the *examining division had failed to provide such substantiation. Also, the passage in the "Case Law of the Boards of Appeal of the EPO" (see paragraph immediately above) could not support its view that in ex parte proceedings the applicant has the burden of proof for facts in his favour, as it appeared to have been taken out of its context. In this respect, the board clarified that all the procedural situations referred to in this passage were those where, in response to a substantiated objection from the examining division, the applicant was required to support its contention.*"

Further, the same section of the Case Law of the Boards of Appeal (III-G-5.1.1) goes on to explain that:

> "*Where a lack of novelty is alleged, the burden of proof invariably lies with the party claiming that the information in question was made available to the public before the relevant date (see, for example **T 193/84**, **T 73/86**, **T 162/87**, **T 293/87**, **T 381/87**, OJ 1990, 213; **T 245/88** and **T 82/90**). According to the boards' established case law, each of the parties to the proceedings bears the burden of proof for the facts it alleges. If a party, whose arguments rest on these alleged facts, does not discharge its burden of proof, this is to the detriment of that party, who may not shift the onus of proof onto the other party (see **T 270/90**, OJ 1993, 725; **T 355/97**; **T 836/02**; **T 176/04**; **T 175/09**; **T 443/09**).*"

We have provided direct evidence of confidentiality agreements in some of the studies using eculizumab (D22, D23 and D31), and declaratory evidence that confidentiality provisions applied to all of the studies using eculizumab (D12, D13 and

D31). The Examining Division, on the other hand, has not substantiated its arguments that eculizumab was made available to the public as a result of the clinical trials carried out before the filing date, nor has the Examining Division provided any contrary evidence to that we have submitted.

The evidence we have provided does not include any confidentiality agreements with the patients taking part in the clinical trials. However, the patients were never in a position to inspect or distribute eculizumab. Eculizumab is administered by intravenous infusion in the clinic (see Declaration of Leonard Bell (D12), paragraph 2 and Declaration of Michael Bombara (D31), paragraph 3). Therefore, the intravenous administration of eculizumab is akin to the implantation of a prototype device (T152/03 and T906/01) and the eculizumab clinical trials can be distinguished from the trials in T7/07 where a large number of patients were given tablets to take home with them for use over a longer period of time (see T7/07 Reason 3.3). The eculizumab used in the trials carried out prior to the filing date of the present application remained under the strict control of health care practitioners who were bound by strict confidentiality obligations and was not made available or accessible to the public.

Further, it is established in T152/03 and T906/01 that the development and test phases of implanted products were necessarily surrounded by secrecy as long as such products had not been commercialised, and that there was a prima facie assumption that any person involved in a medical tests of such products was obliged to maintain confidentiality and that any evidence proving the contrary was important. In the present case, eculizumab had not been commercialised at the filing date (see D13, paragraph 14, D15 and D16). There should therefore be an assumption that those involved with the trials were obliged to maintain confidentiality. Furthermore, we have provided evidence that the trials were carried out under confidentiality agreements with Alexion and that eculizumab used in the clinical trials remained under the strict control of Alexion (see D12, D13 and D31).

As stated in the Case Law of the Boards of Appeal, 8th edition, III-G-4.3.1:

> *"The EPO departments decide on the issues that arise before them on the basis of the evidence adduced by the parties. Their decisions need not, and in most cases cannot, be based on absolute conviction, but instead are to be arrived at on the basis of the overall balance of probabilities, in other words on the footing that one set of facts is more likely to be true than the other. If the result of the boards' evaluation of the evidence is such as to persuade them one way or another, then the* ***balance of probabilities*** *standard is met. This standard applies particularly in opposition appeal proceedings where the boards of appeal are called upon to reach a conclusion on the basis of the overall balance of probabilities, as distinct from "beyond all reasonable doubt" or "absolute conviction" (on the latter concept, refer to the more recent case* ***T 2451/13****). Each of the parties must therefore seek to prove facts alleged by it to that degree of proof (see e.g.* ***T 182/89****, OJ 1991, 391;* ***T 270/90****, OJ 1993, 725;* ***T 859/90****,* ***T 109/91****,* ***T 409/91****, OJ 1994, 653;* ***T 1054/92****,* ***T 296/93****, OJ 1995, 627;* ***T 326/93****,* ***T 343/95****,* ***T 363/96****). The standard of balance of probabilities applies also in decisions issued in ex parte proceedings (****T 381/87****, OJ 1990, 213;* ***T 69/86****,* ***T 128/87****, OJ 1989, 406;* ***T 939/92****, OJ 1996, 309).*

> *When an issue of fact is being examined and decided by the EPO on the balance of probabilities,* ***the more serious the issue*** *the more convincing must the evidence be to support it. If a decision on such an issue may result in refusal or revocation of a European patent, for example, in a case concerning alleged prior publication or prior use, the available evidence in relation to that issue must be very critically and strictly examined. A European patent should not be refused or revoked unless the grounds for refusal or revocation (that is, the legal and factual reasons) are fully and properly proved (**T 750/94**, OJ 1998, 32; **T 329/02**). See also Guidelines G-IV, 1 "State of the art – General remarks and definition" – November 2015 version): "If the applicant shows sound reasons for doubting whether the document forms part of the 'state of the art' in relation to his application and any further investigation does not produce evidence sufficient to remove that doubt, the examiner should not pursue the matter further."* [emphasis added]

The evidence we have submitted shows that the clinical trials using eculizumab were carried out under confidentiality agreements and that Alexion imposed strict controls on the eculizumab used in the trials. The eculizumab used in the trials was not available to the public. There was no public prior use of eculizumab, and the documents that describe the clinical trials that refer to eculizumab only by name (D1-D5, D10 and the patents and applications listed in D8 that have an earlier filing date that the present application) do not provide an enabling disclosure of eculizumab.

Use of the term "eculizumab" is not evidence that it was publicly available

The Examining Division is incorrect that the fact that many patent and non-patent documents in the prior art related to the use of "eculizumab" is evidence that the compound is well-known and available to the public.

It is established Board of Appeal case law that simply mentioning a biological material by name in a document does not make it publicly available. As stated by the Board in T576/91, if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "available to the public" within the meaning of Article 54(2) EPC.

In this case, access to eculizumab was limited to the physicians involved in the trials who had signed confidentiality agreements with Alexion, agreed to store eculizumab in a secure, limited-access area and agreed to strict provisions regarding the tracking and disposal of any unused eculizumab. The mention of "eculizumab" by name in public reports of the trials and in patent applications is therefore not evidence that eculizumab was publicly available.

Thus, the absence of a statement in D1-D5 that eculizumab had not been "*available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals*" does not mean that the antibody was available to the public. The evidence shows that the working group using eculizumab was bound to a confidentiality agreement with Alexion Pharmaceuticals. Therefore,

in accordance with the case law (T576/91), it cannot be concluded that eculizumab had been made "available to the public" within the meaning of Article 54(2) EPC.

Articles 76(1) and 123(2) EPC

The amendment to refer to a light chain consisting of residues 23-214 of SEQ ID NO: 4, and to a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 is an obvious correction that meets with the requirements of Rule 139 EPC.

The Examining Division considers that:

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and the application as originally filed were actually those for which protection was sought."*

The Examining Division notes that neither the original application nor the parent application contain a disclosure of the fact that SEQ ID NOs: 3 and 4 did not have to include the leader peptide. Such an indication is not, however, necessary because it was part of the skilled person's common general knowledge that antibodies do not include leader peptides. Evidence in support of the skilled person's common general knowledge has already been filed as D14, D19 and D20.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "T*he part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC."* (Head note 1; emphasis added)

The Examining Division talks in terms of protection being sought for SEQ ID NOs: 3 and 4. However, protection of these sequences is not what is sought. Rather, it is protection for an antibody defined in terms of its sequences that is sought by the Applicant. Therefore, it is not correction of SEQ ID NOs: 3 and 4 that is requested. Rather, correction is requested only of the definition of an antibody defined with reference to these sequences.

It would have been immediately apparent to the skilled person that the eculizumab light chain sequences shown in SEQ ID NOs: 3 and 4 of the original application and of the parent application include a 22 amino acid signal peptide at the N-terminus. The skilled person would know from his common general knowledge that the 22 amino acid signal peptide in SEQ ID NOs: 3 and 4 would be cleaved off during antibody production in the cell.

The skilled person would consequently immediately recognise that the recitation at page 5, lines 30-33 of the original application and of the parent application of an antibody that comprises a light chain that "*consists of SEQ ID NO: 4*" is an error, and

that the recitation at page 5, lines 25-28 of an antibody having a light chain variable region that "*consists of SEQ ID NO: 3*" is an error.

The skilled person, based on his common general knowledge, would also immediately know that it was the Applicant's intention to define the antibody as including only the mature light chain sequence, i.e. residues 23-214 of SEQ ID NO: 4 or residues 23-109 of SEQ ID NO: 3.

The correction to define the antibody only by reference to the mature light chain sequence is therefore an obvious correction in accordance with Rule 139 EPC, and the present application meets the requirements of Articles 76(1) and 123(2) EPC.

Sufficiency - Article 83 EPC

The insufficiency objection raised by the Examining Division arises because the Examining Division does not accept that the correction made to define the antibody in terms of the mature heavy and light chain sequences is an obvious correction of an obvious error. However, the Examining Division has misinterpreted the Applicant's arguments. The application does not include the wrong sequences. SEQ ID NOs: 3 and 4 are the correct light chain variable and full light chain sequences, respectively, but they contain the 22 amino acid signal peptide that is cleaved off during antibody production and so is not present in the mature antibody which comprises two heavy chains and two light chains. Accordingly, the sequences filed in the original application do identify the structure of eculizumab.

The question to ask when determining whether the disclosure of the application is sufficient is whether the skilled person could make the claimed antibody without undue burden based on the teaching in the patent.

Claim 1 defines the antibody by reference to its full mature heavy and light chain sequences. It is routine for the skilled person to make an antibody having known full mature heavy and light chain sequences. Therefore, there is no question that the skilled person would be able to make the antibody of claim 1 without undue burden.

Inventive Step - Article 56 EPC

The Examining Division has identified the difference between the "*available documents of the prior art describing eculizumab (D1-D5, D10 and D11)*" and the claimed antibody as being the sequence of the claimed compound. The Examining Division refers to the incorrect CAS database entry for eculizumab as was available at the filing date. It is argued that the contribution of the patent is correction of an error in a registered sequence listing, which is devoid of technical character. The Examining Division then asserts that the skilled person had the knowledge to "*carry out the sequence of eculizumab (see the teaching of D7)*". This is not the correct application of the problem and solution approach.

As discussed above, none of D1-D5, D7, D10 and D11 provide an enabling disclosure of "eculizumab". Therefore, the teachings of these documents in so far as they refer to "eculizumab" are not part of the state of the art under Article 54(2) EPC (T1437/05).

The present application is concerned with providing treatments for PNH. Therefore the closest prior art document is one that is also concerned with treating PNH.

D1 is concerned with treating PNH, and other haemolytic diseases, using inhibitors of complement components, such as complement-inhibiting antibodies. D1 describes a clinical trial using the specific anti-C5 antibody "eculizumab". D1 does not disclose the sequence of "eculizumab", but cites D7 and D11 as describing suitable anti-C5 antibodies including "eculizumab". As explained above, D11 does not disclose the full sequence of any antibody and the only full length antibody described in D7 is an IgG4 isotype antibody. Therefore, the teaching in D1 would have led the skilled person to an antibody having a naturally occurring IgG4 heavy chain constant region. Thus, whilst D1 does not provide an enabling disclosure of "eculizumab" for treating PNH, it does provide an enabling disclosure of the IgG4 antibody disclosed in D7 for treating PNH.

In contrast, the antibody of claim 1 has an engineered heavy chain constant region. The amino acid differences between the IgG4 heavy chain constant region and the engineered heavy chain constant region of the antibody of claim 1 are shown in the Bell declaration (D12) in paragraph 5. The technical effects of these differences are also set out in D12. Paragraph 6 of D12 explains that differences in the heavy chain constant region result in the antibody having reduced binding to Fcγ receptors such that immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway are minimised (see also the experimental report, D18). Paragraph 7 of D12 explains that differences in the heavy chain constant region result in the antibody having an increased plasma half-life (see also the experimental report attached as D29).

The objective technical problem in view of D1 may therefore be defined as the provision of an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH. The solution is solved by the presently claimed antibody. The solution provided would not have been obvious to the skilled person at the filing date.

D7 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> "*The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)*" (D7, page 1399, right column, second paragraph, lines 15-25)."

The skilled person would have had no motivation to use an antibody different than the IgG4 antibody described in D1/D7 in view of the report in D1 of the successful treatment of PNH, and the teaching in D7 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only

one known allotype, precluding an immune response against the constant region of the antibody.

In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D7 because he would be concerned about the immunogenicity of the therapeutic antibody, and D7 teaches that the IgG4 isotype is ideal in this regard. The safety of the IgG4 antibody of D7 would have been confirmed to a skilled person by the clinical studies reporting that "eculizumab" (identified as the antibody of D7) was safe and effective when administered to PNH patients (e.g. D1, D3 and D4). Also, at the filing date of the application, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D30, table 1). Significantly, at the filing date of the application, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market or publicly known to be studied in human clinical trials.

Furthermore, the advantageous properties of reduced immunogenicity and increased half-life exhibited by the claimed antibody are not taught or suggested in any of the cited documents. Indeed, in paragraph 8 of D12, it is explained that the advantageous properties of the claimed antibody over the antibody of D7 (which is the antibody to which D1 relates) were surprising and unpredictable.

Accordingly, the solution to the problem of providing an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH provided by the present claims would not have been obvious to the skilled person at the filing date, and the claims involve an inventive step.

First Auxiliary Request

In the first Auxiliary Request, the antibody is defined as "*comprising the heavy chain amino acid shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4*". Basis for this amendment may be found in the originally filed application at page 4, line 32 and page 44.

Second Auxiliary Request

The second Auxiliary Request is the same as the Main Request, except that claim 1 has been rewritten as a purpose-limited product claim reciting the treatment of PNH.

Third Auxiliary Request

The third Auxiliary Request contains the amendments made to the first and second Auxiliary Requests.

Conclusion

It is believed that this application is in allowable form and the Examining Division is requested to reconsider it favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. The Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be

allowed without the need for Oral Proceedings on 5 September 2019, on the basis of the Main Request or, failing that, on one of the Auxiliary Requests. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

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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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 Mailose, 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)	Arcitumomab; 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 Mannniol	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

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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*)

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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 Yttrium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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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

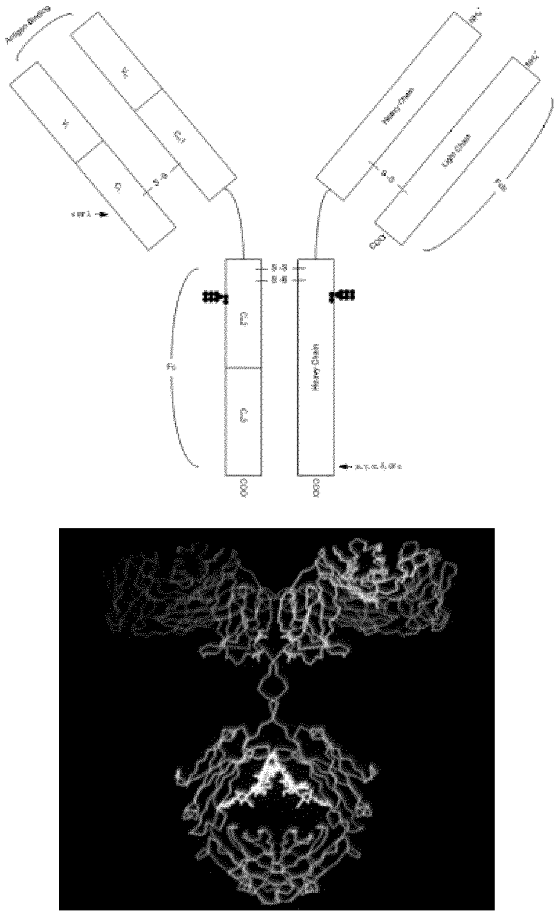


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

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

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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

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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

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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

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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)

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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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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

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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 iso-aspartate).[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

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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]

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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

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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

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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

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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

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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

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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

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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

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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,

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and (3) minimization of antibody-formulation-induced immune response.

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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Letter dealing with Oral proceedings	Final Written Submissions N411599EP-B PXT.PDF	ORAL-1.PDF
2	Amended claims (clean copy)	Auxiliary Request 1 (clean) N411599EP-B PXT.PDF	CLMS-1.PDF
3	Amended claims with annotations	Auxiliary Request 1 (tracked) N411599EP-B PXT.PDF	CLMS-HWA-1.PDF
4	Amended claims (clean copy)	Auxiliary Request 2 (clean) N411599EP-B PXT.pdf	CLMS-2.pdf
5	Amended claims with annotations	Auxiliary Request 2 (tracked) N411599EP-B PXT.pdf	CLMS-HWA-2.pdf
6	Amended claims (clean copy)	Auxiliary Request 3 (clean) N411599EP-B PXT.PDF	CLMS-3.PDF
7	Amended claims with annotations	Auxiliary Request 3 (tracked) N411599EP-B PXT.PDF	CLMS-HWA-3.PDF
8	Letter dealing with Oral proceedings	Cited Documents N411599EP-B.PDF	ORAL-2.PDF
9	Letter dealing with Oral proceedings	D24.pdf	ORAL-3.pdf
10	Letter dealing with Oral proceedings	D25 Summons to OPs on EP1720571 - N411599EP-B.PDF	ORAL-4.PDF
11	Letter dealing with Oral proceedings	D26 Alexion response to Summons on EP1720571 - N411599EP-B.PDF	ORAL-5.PDF
12	Letter dealing with Oral proceedings	D27 Decision revoking EP1720571 - N411599EP-B.PDF	ORAL-6.PDF
13	Letter dealing with Oral proceedings	D28 - Annotated CAS Registry.pdf	ORAL-7.pdf
14	Letter dealing with Oral proceedings	D28A - Annotated CAS Registry.pdf	ORAL-8.pdf
15	Letter dealing with Oral proceedings	D29 Experimental report N411599EP-B.PDF	ORAL-9.PDF
16	Letter dealing with Oral proceedings	D30 - Wang et al..pdf	ORAL-10.pdf

17	Letter dealing with Oral proceedings	D31 Bombara Declaration.PDF	ORAL-11.PDF

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Oxford, GB**
Date:	**05 July 2019**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp**
Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

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AUXILIARY REQUEST 2
CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 1
CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST 3
CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

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Date: 01-07-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Decision revoking the European patent (Art. 101(2), (3)(b) EPC)

The Opposition Division - at the oral proceedings dated 15.05.14 - has decided:

The European Patent No. 1720571 is revoked.

☐ Additional decision:
The opposition of the opponent(s) ____ is rejected as inadmissible.
Apportionment of costs (Art. 104(1) EPC) as indicated in the grounds for the decision.

The reasons for the decision are enclosed.

Appeal
This decision is open to appeal. Attention is drawn to the attached text of Articles 106 to 108 EPC and Rules 97 and 98 EPC.

Opposition Division:

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

Enclosures: Reasons for the decision (Form 2916, 10 pages)
Text of Articles 106 - 108 and Rules 97 - 98 EPC (EPO Form 2019)
☒ Minutes of oral proceedings

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EPO Form 2331 12.07 (26/06/14) REVO

to EPO postal service: 26.06.14

CC20871

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

D13 Kaplan, (2002) Current Opinion in Inv. Drugs, vol. 3, no. 7, pages 1017-1023

D14 Declaration of Leonard Bell (2014)

D15 Packaging insert/Datasheet from Eculizumab (Alexion, 2011)

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed. The P requested oral proceedings in case their respective request would not be granted.

1.6 The Opposition Division (OD) issued a non-binding preliminary opinion with their official communication of 14.01.2014, based on the grounds of opposition raised by the O, and which the OD considered pertinent to pursue of its own motion, in accordance with Article 114 EPC. Oral proceedings were appointed for 15.05.2014.

1.7 The P submitted document D13 and arguments in support of maintenance of the patent as granted with their letter of 30.12.2013.

1.8 The OD reissued their non-binding preliminary opinion in order to account for the P's arguments that had not been presented to the OD before issuance of the summons to oral proceedings. The date scheduled for oral proceedings was maintained.

1.9 In their letter of 17.013.2014, the P submitted further arguments, a new main request (MR) and an auxiliary request (AR1) to replace the claims of the opposed patent. The P requested maintenance of the scheduled oral proceedings, in case the OD still not consider that any of the new requests overcome the objections raised.

1.10 Oral proceedings took place on 15.05.2014 in the presence of the P alone, since the O had withdrawn from proceedings. At the proceedings the Chairman (C) informed the P that the examination of the opposition was thus being continued of the OD's own motion. The P maintained their requests on file and submitted a declaration (D14), and a datasheet (D15) to support the subject-matter claimed.

1.11 At the end of oral proceedings, the OD announced the decision to revoke the patent in full in the absence of further allowable requests, because AR1 did not fulfill the requirements of Article 56 EPC.

2 Reasons for the Decision

Admissibility

2.1 The opposition complied with Articles 99 and 100 EPC. No objections were raised. The opposition is allowable.

Procedural matters

2.2 The O formally withdrew their opposition to the patent on 07.10.2013.

2.3 The OD considered that the arguments and documents submitted by the O to be valid grounds for opposition and thus decided to pursue opposition *proceedings of its own motion* (Article 114(1) EPC; GL D, VII-5.2;5.3).

2.4 The P filed D14, a declaration of a technical expert, and D15, a datasheet, during oral proceedings. The OD decided to admit D14 and D15 in to the proceedings, since they provided background information to the subject-matter claimed.

The decision is being carried out on the **following requests:**

Main Request

Description, Pages

3-13	of the patent specification

Claims, Numbers

1-8	received on	18-03-2014	with letter of	18-03-2014

Drawings, Sheets

1/11-11/11	of the patent specification

Auxiliary Request 1

Description, Pages

3-13 of the patent specification

Claims, Numbers

1-8 received on 18-03-2014 with letter of 18-03-2014

Drawings, Sheets

1/11-11/11 of the patent specification

Main Request (MR)

The MR differs from the patent specification through the modification of the route of administration to explicitly state 'intravenous infusion', thus replacing the term 'intravenously' in claim 1 of the granted patent.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.5 The OD decided that the MR complies with Rule 80, Articles 84, 123(2) and 123(3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. All objections raised were thus overcome.

Lack of novelty (Articles 100(a) and 54 EPC)

2.6 Objections were raised on grounds of lack of novelty of claims 1 and 5 with regard to D1.

2.7 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.8 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.9 The OD considered that since the priority document discloses the claimed dosage regimen for treatment of complement breakthrough via intravenous infusion, D1 is not considered prior art and the subject-matter of claims 1 and 5 is new.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.10 The OD objected to the MR on grounds of a lack of inventive step, in light of D5 alone as the closest prior art to the subject matter of claims 1 and 5, or in combination with any of D3, D4 or D6.

2.11 The P concurred that D5 should be considered the closest prior art, as it is directed to the same technical purpose, i.e. it is directed to treating a patient afflicted with PNH suffering a breakthrough in hemolytic activity.

2.12 Document D5 discloses the continuation of a pilot study comprising:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 900mg bi-weekly
5. reducing the dosing interval slightly in response to breakthrough and thus resolving the symptoms of PNH.

2.13 The P stated that the difference between claim 1 and document D5 can therefore be considered to be that D5 does not disclose a complete dosage schedule. D5 is directed only to the maintenance stage of treatment, i.e. stages (iii) and (iv) of claim 1, and does not teach the essential induction phase stages (i) and (ii).

2.14 The technical effect associated with this difference is a complete dosage schedule for treating PNH and modifications for the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.15 The problem to be solved can therefore be considered to be the provision of a complete dosage regimen for maintaining patients with PNH symptom free.

2.16 The solution provided in the granted claims is not obvious, since the essential pre-loading stage is not taught in D5.

2.17 The P referred to the declaration D14, §4, wherein it is stated that the administration of 900 mg eculizumab at week five is essential for achieving an adequate baseline of complement inhibition that can be maintained with bi-

weekly 900 mg eculizumab doses thereafter. The P referred to the dosage regimen and provisions for its use, stated in the packaging datasheet D15 that accompanies eculizumab, as proof that this is the regimen used in practice.

The P thus concluded that D5 alone does not teach the features necessary to provide a complete dosage regimen for maintaining patients with PNH symptom free.

2.18 Furthermore, the P stated that D5 combined with any of D3,D4 or D6 would not permit the skilled person to arrive at the regimen claimed:

Documents D3 and D4 disclose a pilot study comprising the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg bi-weekly thereafter

The skilled person combining the teaching of D5 with any of D3 or D4 would not arrive at the dosage regimen claimed, since the administration of 900 mg eculizumab at week 5 is not taught.

2.19 The P explained that the skilled person would not arrive at the claimed dosage regimen when combining the teaching of D5 with D6:

Document D6 discloses the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.20 D6 teaches the administration of 900 mg eculizumab at week 5. However, the skilled person following the dosage schedule of D6 and experiencing breakthrough of hemolytic activity in PNH patients would not reduce the dosage interval to 900 mg eculizumab every 12 days: D5 merely teaches the 'slight' reduction of the dose in this situation. The P claimed that a reduction in dosage interval from 14 to 12 days cannot be considered slight, as it amounts

to around 15%. Moreover, the skilled person with a knowledge of D13 (p. 1021, col.1, §1 - 4) would know that the behaviour of the complement system is difficult to predict, and would not reduce the intervals by so much, and certainly not to 12 days, since shortening of dosing intervals may not result in an effective treatment by eculizumab.

2.21 The OD argued that the replacement of one regimen with another is a matter of routine experimentation, as supported by case law of the Technical Board of Appeal T1979/09, wherein under point 7.3.1 it is stated *'It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.'*

and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.'*

2.22 The P disagreed with this interpretation, since the present regimen comprises 4 different steps as stated in the claim, and thus cannot be compared to the simple regimen considered in T1979/09.

2.23 The OD argued that D13 is not directed to the same technical problem posed. Claim 1 is clearly directed to patients suffering breakthrough who are already receiving a standard regimen, as disclosed in D6. D5 clearly discloses how cases in which breakthrough is experienced can be resolved. D5, alike D6 was published after D13 and represents the actual stand of knowledge at the time of priority filing. The OD therefore considers that given the nature of D13 in reporting preliminary research results, and the subsequent publications D5 and D6, the skilled person would not consider D13 when selecting a dosage regimen for the indication presently claimed.

2.24 The P argued that neither D5 alone or combined with D6 any other cited document would anticipate the subject-matter of claim 1, however, if the skilled person were to combine D5 with other documents, he would select D3 or D4 as these relate to less complicated teachings.

2.25 The OD considers D6 to be the most recent and detailed teaching of the cited prior art D3,D4 and D6. The skilled person would therefore not consider D6 too complicated, rather to be the most up-to-date teaching for the treatment of PNH based on the pilot studies described in D2,D3 and D4.

2.26 The OD finds that according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D6, were known to be preventable by a slight decrease in the dosing interval. The skilled person would have chosen and combined the teachings of D5 and D6 because they both describe the treatment of PNH with eculizumab. D6 discloses the latest details of a pilot study comprising the dose loading stages of parts (i) - (iii) of claim 1, whereas D5 describes an extension to what appears to be the same pilot study, wherein patients experiencing breakthrough of hemolytic activity are treated by a slight reduction in dosing interval, which successfully resolves their symptoms.

2.27 The OD thus concludes that the skilled person would be taught by D5 to reduce the dosage interval to solve the problem of breakthrough in PNH patients receiving the priming dosage regimen described in D6. The skilled person would reduce the interval 'slightly', i.e. by at say least 10%, to 12 days to resolve the symptoms, as suggested by D5.

The subject-matter of the MR therefore does not meet the requirements of Article 56 EPC.

Auxiliary Request 1 (AR1)

AR1 differs from the MR in that a result to be achieved by the dosage regimen of claim 1 of the MR has been added, i.e. a reduction of serum hemolytic activity (cell lysis) below 20%.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.28 The OD decided that AR1 complies with Rule 80, Articles 84, 123(2) and 123 (3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. The feature that cell lysis is reduced by at least 20% was deemed to find a basis on p.38, li.11-17.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.29 The OD objected to AR1 on grounds of a lack of inventive step, in light of D5 as the closest prior art to the subject matter of claims 1 and 5, in combination with D6.

2.30 The P argued that neither D5 nor D6 refer to any threshold value to be used to assess breakthrough of hemolytic activity.

2.31 The problem to be solved may thus be considered to be the provision of a dosage regimen that permits the maintenance of complement activity below a threshold that prevents breakthrough.

2.32 The solution is the dosage regimen of AR1. Neither D5 or D6 teach how to assess breakthrough by the parameters claimed. The application clearly discloses how serum hemolytic activity can be used to identify patients experiencing breakthrough, and that reducing this activity below 20% correlates resolves the symptoms of PNH (p.38 and Fig.8).

2.33 The OD noted that Fig.8 shows at least 4 patients with hemolytic activity above 20%, yet only 2 are described as experiencing breakthrough. The claimed parameter would thus appear to be a non-technical feature of the claim whose relevance is unclear, since patients with serum hemolytic activity above this threshold do not necessarily appear to demonstrate breakthrough.

2.34 The P explained that classic symptoms such as urine colour (hemoglobinuria) were signs of hemolytic breakthrough. In D5, LDH, hemoglobinuria and other mild symptoms were measured, but these are not direct methods of measuring serum complement activity. The parameter claimed thus identifies patients that are successfully treated.

2.35 The OD finds that the technical effect of the dosage regimen claimed is the treatment of PNH. The claim is directed to a dosage regimen and not to a method of diagnosis.

Fig.8 of the application demonstrates that patients with 0 - 30% serum hemolytic activity do not exhibit symptoms of PNH in the group tested. Not all patients are maintained below 20% activity, suggesting that the regimen claimed may not even achieve the technical effect claimed.

Furthermore, alternative methods for monitoring complement breakthrough are disclosed in Fig.4 of the application, which are the same as those used in D5.

The parameter of reducing hemolytic activity below 20% does not appear to provide a further technical feature associated with any surprising technical effect, but may at best be another read-out to confirm the same resolution of breakthrough already recorded in D5.

2.36 The OD therefore concludes that AR1 does not meet the requirements of Article 56 EPC.

Decision

3 Taking into account the amendments made by the P, the patent according to the Main Request or Auxiliary Request 1 was found not to comply with the requirements of Article 56 EPC. The OD decided to revoke the amended patent in accordance with the provisions of Article 101(3)(b) EPC.

D28

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Substance Details

CAS Registry Number: 219685-50-4

CA Index Name: Immunoglobulin, anti-(human complement C5 α-chain) (human-mouse monoclonal 5G1.1 heavy chain), disulfide with human-mouse monoclonal 5G1.1 light chain, dimer

Sequence Strand: 1728, 650, 650, 214, 214

```
1:    QVQLVQSGAE VKKPGASVKV SCKASGYIFS NYWIQWVRQA PGQGLEWMGE
51:   ILPGSGSTEY TENFKDRVTM TRDTSTSTVY MELSSLRSED TAVYYCARYF
101:  FGSSPNWYFD VWGQGTLVTV SSASTKGPSV FPLAPCSRST SESTAALGCL
151:  VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSNFGT
201:  QTYTCNVDHK PSNTKVDKTV GERPAQGGRV SAGSQAQPSC LDAPRLCSPS
251:  PGQQGRPHLS PHPEASARPT HAQGEGLLAF STRLQAGTGW VPLPQALHTQ
301:  GQVLGSDLPK AISGRTLPPD LSRPQGQTVH SLSSDTFLSS QIRVTPNLLS
351:  AERKCCVECP PCPGKPAQAS PSSSRRDRCP RVACIQGQPQ LGADTSTSIS
401:  SSAPPVAGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
451:  DGVEVHNAKT KPREEQFNST DRVVSVLTVL HQDWLNGKEY KCKVSTKASR
501:  PPSRKPSPKP KVGPTGCEGH MDRGQLGPPS ALGVTAVPTS VPTGQPREPQ
551:  VYTLPPSQEE MTKNQVSLTC LVKGLYPSDI AVEWESNGQP ENNYKTTPPV
601:  LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV MHEALHNHYT QKSLSLSLGK

1:    QVQLVQSGAE VKKPGASVKV SCKASGYIFS NYWIQWVRQA PGQGLEWMGE
51:   ILPGSGSTEY TENFKDRVTM TRDTSTSTVY MELSSLRSED TAVYYCARYF
101:  FGSSPNWYFD VWGQGTLVTV SSASTKGPSV FPLAPCSRST SESTAALGCL
151:  VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSNFGT
201:  QTYTCNVDHK PSNTKVDKTV GERPAQGGRV SAGSQAQPSC LDAPRLCSPS
251:  PGQQGRPHLS PHPEASARPT HAQGEGLLAF STRLQAGTGW VPLPQALHTQ
301:  GQVLGSDLPK AISGRTLPPD LSRPQGQTVH SLSSDTFLSS QIRVTPNLLS
351:  AERKCCVECP PCPGKPAQAS PSSSRRDRCP RVACIQGQPQ LGADTSTSIS
401:  SSAPPVAGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
451:  DGVEVHNAKT KPREEQFNST DRVVSVLTVL HQDWLNGKEY KCKVSTKASR
501:  PPSRKPSPKP KVGPTGCEGH MDRGQLGPPS ALGVTAVPTS VPTGQPREPQ
551:  VYTLPPSQEE MTKNQVSLTC LVKGLYPSDI AVEWESNGQP ENNYKTTPPV
601:  LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV MHEALHNHYT QKSLSLSLGK

1:    DIQMTQSPSS LSASVGDRVT ITCGASENIY GALNWYQRKP GKAPKLLIYG
51:   ATNLADGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQN VLNTPLTFGQ
101:  GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151:  DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201:  LSSPVTKSFN RGEC

1:    DIQMTQSPSS LSASVGDRVT ITCGASENIY GALNWYQRKP GKAPKLLIYG
51:   ATNLADGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQN VLNTPLTFGQ
101:  GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151:  DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201:  LSSPVTKSFN RGEC
```

Y

NKQLPSSIEKTISKAK

F

Q

Sequence Notes:

Type	Location	Description
bridge	CYS-96 CYS-22	disulfide bridge
bridge	CYS-205 CYS-149	disulfide bridge
bridge	CYS-96' CYS-22'	disulfide bridge
bridge	CYS-205' CYS-149'	disulfide bridge
bridge	CYS-88" CYS-23"	disulfide bridge
bridge	CYS-194" CYS-134"	disulfide bridge
bridge	CYS-136 CYS-214"	disulfide bridge
bridge	CYS-88"' CYS-23"'	disulfide bridge
bridge	CYS-194"' CYS-134"'	disulfide bridge
bridge	CYS-136' CYS-214"'	disulfide bridge
bridge	CYS-240 CYS-240'	disulfide bridge

—— underlined amino acids are deleted in the correct sequence

▭ boxed amino acids are substituted in the correct sequence as shown in the boxes on the right of the sequences

Type	Location	Description
bridge	CYS-247 CYS-247'	disulfide bridge

Chemical Structure:

Registry Number: 219685-50-4
Formula: Unspecified

No Structure Diagram Available

(disclaimer)

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DECLARATION BY MICHAEL BOMBARA

I, Michael Bombara, Executive Director, Clinical Business Operations – R&D, of Alexion Pharmaceuticals, Inc., of 121 Seaport Boulevard, Boston, MA, United States of America, make the following factual declaration concerning the clinical trial practices at Alexion in the late 1990s and early 2000s when Alexion conducted several clinical studies using eculizumab:

1. I am Executive Director, Clinical Business Operations – R&D, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which is attached as Exhibit A.

2. I obtained my Bachelor of Science degree in Microbiology in 1984 from the University of Maine. Thereafter, I held laboratory scientist positions of increasing responsibility from 1984 through 1996 at Genentech Incorporated and Bayer Corporation. In 1996 when I became a Medical Research Associate at Bayer, my main work responsibilities began focusing on day-to-day clinical trial management. Two years later I joined Alexion as a Clinical Project Leader. From 1998 through the present I have been working in the Clinical Operations group at Alexion in various roles: from a Clinical Project Leader in 1998 to a Clinical Project Manager in 2001 to now a Clinical Business Operations Lead, which is the role I have held since 2013. I have lead more than a dozen clinical trials at Alexion for several investigative drugs in various indications. Through this work, I have been involved in the day-to-day clinical study-related activities at Alexion, including working with principal investigators and clinical study sites in order to get studies started and make sure they are run in accordance with Alexion's best practices (and also in accordance with all applicable laws and regulations). My responsibilities have included ensuring that all of the necessary contracts between Alexion and the principal investigators and/or their institutions or study sites are in place and making sure that each study protocol is signed by all participating investigators. As I was working as a Clinical Project Manager at Alexion in the late 1990s and early 2000s, I managed many of the clinical trials that Alexion conducted using eculizumab during that time-frame, including a pilot study in which patients afflicted with paroxysmal nocturnal hemoglobinuria ("PNH") were treated with eculizumab, as well as two extension studies thereof.

3. I understand that D1 to D5 are literature references that the European Patent Office has referred to in its "Summons to attend oral proceedings" dated March 27, 2019. I have reviewed each of these documents and explain below how the studies described in each of them were carried out in a manner in which eculizumab and information relating thereto, could not have been disclosed to or used by the public due to: (1) strict confidentiality provisions (in not one but at least two separate agreements governing each study) that each physician and his/her institution involved in the study were bound to abide by; and (2) strict drug accountability provisions in each study protocol that each investigator and institution were bound by, including the requirement that the study drug

eculizumab be stored in only a secure, limited-access storage area. In addition, given that eculizumab has always been administered intravenously, in all of these clinical studies the eculizumab was administered to each patient at a healthcare facility by a healthcare professional who was bound by the clinical study protocol; this is not a case where the patient could have administered the drug to himself, at home, or would have ever had an opportunity to take the drug out of the facility where he was treated. Attached as Exhibit B is also a summary of references D1 to D5 showing the clinical study or studies that each reference describes and whether participants in those studies were bound to keep Alexion's information and drug substance confidential.

4. For many of the studies described in D1 to D5 above, I participated first-hand in ensuring that all of the necessary agreements were in place before a study investigator received any confidential Alexion information and before the clinical study site received any study drug (eculizumab). Alexion was extremely careful in maintaining confidentiality around each ongoing trial and the drug substance used therein (eculizumab).

Confidentiality Regarding Each Clinical Study

5. It was Alexion's clinical trial practice in at least the late 1990s and early 2000s to have multiple layers of confidentiality built in to each clinical study to ensure that the participating investigators and study institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence. For example, Alexion typically entered into the following types of agreements for each study:

- Master Clinical Study Agreement ("MCSA"): Alexion entered into this Agreement with an institution or site that it intended to participate with to carry out one or more clinical studies. This Agreement set forth the general terms – including confidentiality requirements – that the parties agreed to abide by in cooperating with one another in the course of performing the study.

- Exhibit to the MCSA: An exhibit to the MCSA referenced the particular clinical study that the parties agreed to work together on, and set forth that the parties' work on that study was governed by the overall provisions of the MCSA, specifically including the confidentiality provisions thereof.

- Clinical Study Protocol: Once the MCSA was in place and the study site and physicians began working with Alexion to carry out a study, Alexion shared with the site and the physicians the Clinical Study Protocol, which set forth the exact procedure that the participating physician agreed to follow in conducting the study. The Clinical Study Protocol included requirements of confidentiality as well as requirements that the physical study drug be kept in a secure, limited-access location and be carefully tracked via a specific drug accountability logging procedure.

- Consulting Agreement: Often times Alexion also had a previous or co-existing consulting agreement in place with the physicians participating in the clinical study. This agreement set forth the terms that Alexion and the physician agreed to abide by in working with one another to discuss one another's thoughts and ideas as well as study results. The consulting agreement included another requirement that the physician maintain all of Alexion's proprietary information in confidence.

6. As one specific example of how all of these agreements fit together in one particular study, the pilot PNH study of 11 patients that is referenced in D1 to D5 had several layers of confidentiality built into it. (This pilot study is referred to as the "C02-001 Study.") First, there was a Master Clinical Study Agreement ("MCSA") in place between Alexion and each participating institution or study site that required the institution to maintain Alexion's information, which included information about the study drug eculizumab, in strict confidence. As an example, attached as Exhibit C is the MCSA governing C02-001 that was entered into between Alexion and Leeds Teaching Hospitals in the UK, a participant in the C02-001 study. Paragraph 7 of the MCSA states:

   "Institution agrees, and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study 1) information that is disclosed by or on behalf of Alexion to Institution or the relevant Principal Investigator and 2) data, inventions, and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug....Institution agrees to return confidential information of Alexion, and all copies thereof, at the conclusion of each Study or at an earlier date at the request of Alexion..."

   The MCSA also states that the confidentiality provisions "shall survive the termination or expiration of this Agreement."

7. In addition to the express language of the MCSA, Exhibit II to the MCSA (attached as Exhibit D) further describes Study C02-001 that is being conducted pursuant to the MCSA and sets forth additional conditions regarding the Study. Paragraph 8 of this Exhibit specifically references the confidentiality provisions of the MCSA to again highlight these obligations to the signing investigator and his/her institution. Paragraph 8 states: "The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated April 1, 2002...Principal Investigator agrees to...the confidentiality provisions of Section 7 of the Master Clinical Study Agreement."

8. Providing a second separate layer of confidentiality requirements, the clinical trial protocol itself, which every investigator participating in the study had to sign and agree to follow, contained strict confidentiality provisions. The protocol governing the C02-001 Study is attached as Exhibit E. Not only is the protocol itself marked "CONFIDENTIAL," but Section 18 of the protocol states:

"THIS CONFIDENTIAL INFORMATION ABOUT AN INVESTIGATIONAL DRUG IS PROVIDED FOR THE EXCLUSIVE USE OF INVESTIGATORS OF THIS DRUG AND IS SUBJECT TO RECALL AT ANY TIME. THE INFORMATION IN THIS DOCUMENT MAY NOT BE DISCLOSED UNLESS SUCH DISCLOSURE IS REQUIRED BY FEDERAL OR STATE LAW OR REGULATIONS SUBJECT TO THE FOREGOING. THIS INFORMATION MAY BE DISCLOSED ONLY TO THOSE PERSONS INVOLVED IN THE STUDY WHO HAVE A NEED TO KNOW WITH THE OBLIGATION NOT TO FURTHER DISSEMINATE THIS INFORMATION. THESE RESTRICTIONS ON DISCLOSURE WILL APPLY EQUALLY TO ALL FUTURE ORAL OR WRITTEN INFORMATION SUPPLIED TO YOU BY ALEXION, WHICH IS DESIGNATED AS 'PRIVILEGED' OR 'CONFIDENTIAL'."

9. Even further, Alexion often had a Confidential Disclosure Agreement ("CDA") in place with the study investigators. For example, Alexion had a CDA in place with Peter Hillmen of Leeds Teaching Hospitals during Study C02-001. This CDA is attached as Exhibit F. Paragraph 2 of the CDA states:

"The Receiving Party agrees not to (1) disclose to any third party any or all of the Information, or permit any third party to have access to such information, or (2) use such Information for any purpose other than to evaluate the Information in connection with discussions between the parties. In addition, the Receiving Party agrees to disclose the Information only to those of its employees who shall reasonably need to know the information in order to evaluate the Information and who are bound to terms of confidentiality that are substantially as protective as the terms hereof."

10. Additionally, Alexion and the institution participating in the clinical study (or the organization providing the institution's financial support) also typically signed a "Form of Indemnity for Clinical Studies" that again acknowledged that the investigators at the institution were required to carry out the study in accordance with the study protocol which, as explained above, included strict confidentiality requirements. Attached as Exhibit G is this Form of Indemnity for Clinical Studies between Alexion and The Leeds Teaching Hospitals NHS Trust regarding the C02-001 Study.

11. Thus, for the C02-001 Study, with respect to Dr. Peter Hillmen and his institution (Leeds Teaching Hospitals), there were at least five times that confidentiality was required, or the investigator and/or study site were reminded of the required confidentiality terms, before the investigator and/or the site were provided with the eculizumab study drug: in the MCSA, Exhibit II to the MCSA, the C02-001 Study protocol, a separate CDA with Dr. Hillmen, and the Form of Indemnity.

Restricted Access to Study Drug

12. Not only were the investigators and institutions that participated in clinical trials repeatedly legally bound to maintain the confidentiality of Alexion's information, but the investigators and institutions also agreed to abide by the strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material such as eculizumab. For example, as shown in Section 6.6 of the clinical trial protocol for the C02-001 Study (Exhibit E), the receiving investigator or institution was required to maintain an original Receipt of Shipment form upon receipt of study drug material, and then complete a drug accountability ledger that was included in each drug shipment in order "to assist the pharmacist in maintaining current and accurate inventor records covering receipt, dispensing, and the return of study drug supplies." The log required: the identification numbers and initials of subjects to whom the drug was dispensed, the date(s) the drug was dispensed to the subjects, and the date(s) and the number of returned drug vial. Empty vials and vials with residual materials, as well as the inventory records, were required to be kept for later inspection. In addition, all used, partially used and unused vials were required to be documented in terms of how they were disposed of on-site. For example, Exhibit H shows a sample "Drug Disposal Form," which served to document how much drug substance material remained after each administration and/or trial and certified that the institution properly destroyed of any remaining material, e.g., via incineration.

13. In addition, each protocol specified that the study drug be stored in an area with restricted access. For example, Section 6 of the protocol for the C02-001 Study states that, "[a]s with all investigational drugs, Eculizumab must be stored in a secure, limited access storage area." This careful tracking and limited-access storage that was required for all of the eculizumab study drug that Alexion provided for the study ensured that no eculizumab made its way out of the hands of those conducting the study (and who were bound to keep all Alexion information in strict confidence).

14. The C02-001 Study was conducted with 11 PNH patients at two institutions – Leeds Teaching Hospitals and St. George's Medical School, both in the UK. Above I have provided in Exhibits C-G the various documents binding Leeds Teaching Hospitals and its investigators to strict confidentiality and drug accountability and storage provisions. I now attach the corresponding exhibits for the second institution, St. George's Medical School: Exhibit II to the MCSA (attached as Exhibit I, which itself also indicates that an MCSA with similar confidentiality provisions was in place between Alexion and St. George's Medical School) and the Form of Indemnity for Clinical Studies (attached as Exhibit J), in which the St. George's Medical School agreed to abide by the provisions of the C02-001 Study protocol, including the confidentiality and drug accountability and storage provisions I have explained above.

15. I note that all of the authors on references D1, D2, D3 and D5 were affiliated with either Alexion (Leonard Bell, Russell Rother, Scott Rollins, Christopher Mojcik, M. Bombara and B. Petro), Leeds Teaching Hospitals in the UK (Peter Hillmen, Anita Hill, C. Hall, M. Cullen and S.J. Richards), or St. George's Hospital Medical School in the UK (J.

Marsh, D. Elebute and J. Chan). Thus, all authors on these references were bound at least by their own employment agreement confidentiality requirements (at Alexion), or by at least the confidentiality obligations that their respective institutions agreed to abide by in an MCSA. D4 is more of a review article summarizing all of the studies that had been conducted using eculizumab, and the author indicated that the "Source" for the paper was Alexion Pharmaceuticals, Inc.

16. While I have only provided the documents relating to the C02-001 Study, similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s based on my experience and knowledge of Alexion's best practices in clinical study initiation and maintenance at that time. I am simply using C02-001 as a representative example in this declaration.

17. Given the multiple layers of confidentiality requirements governing each eculizumab-related clinical trial in the late 1990s and early 2000s, the requirements of all institutions and their principal investigators to account for all study drug material and maintain such material in a secure, limited-access storage area, and the nature of the administration of eculizumab such that patients would never have had direct access to the drug, no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of these clinical studies.

DECLARED at New Haven, CT
United States of America

This 2nd Day of July 2019

Michael Bombara

CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

EMPLOYMENT HISTORY

Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	May 2017 to Present
Title	Executive Director, Clinical Business Operations Lead
Current Manager	Moke Sharma
Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	March 2016 to May 2017
Title	Clinical Business Operations Lead
Current Manager	Moke Sharma
Current Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	October 2013 to Present
Title	Clinical Business Operations Lead
Current Manager	Mike Collins
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	July 2008 to October 2013
Title	Senior Director, Global Clinical Operations
Manager	Camille Bedrosian and Mike Collins
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	January 2007 to July 2008
Title	Director, Clinical Operations
Manager	Stephen Squinto and Camille Bedrosian
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	August 2004 to January 2007
Title	Associate Director, Clinical Operations
Manager	Christopher Mojcik
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	July 2001 to August 2004
Title	Clinical Project Manager
Manager	Christopher Mojcik
Name of Employer	Alexion Pharmaceuticals, Inc.
Dates of Employment	October 1998 to July 2001
Title	Clinical Project Leader
Manager	Christopher Mojcik

Project Lead for the Following Clinical Studies:

ALXN1210 APAC Regional Lead

ALXN1210 Clinical Operations Lead June 2015 – April 2016

C11-001: An Open Label, Multi-Center Trial of Eculizumab in Patients with Shinga-Toxin producing Escherichia Coli Hemolytic-Uremic Syndrome (STEC-HUS)

E05-001: A Phase 3, Open-label, Extension Study of Eculizumab in Patients With Transfusion Dependent, Hemolytic Paroxysmal Nocturnal Hemoglobinuria (PNH) Who Have Participated in the TRIUMPH (C04-001), SHEPHERD (C04-002), or X03-001 Studies

C04-002: SHEPHERD: Safety in Hemolytic PNH Patients Treated With Eculizumab: A Multicenter Open-label Research Design Study

Samsung Bioepis Exhibit No. 1028
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CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

C04-001: TRIUMPH: A Hemoglobin Stabilization and Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria Patients

E02-001: An Open-label Extension Study of h5G1.1-mAb (Eculizumab) in Patients With Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria Who Previously Participated in Study C02-001

C02-001: An Open-Label Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion-Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria

E01-004: Final Report for a Phase 2b Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide

C01-004: Final Report for a Phase 2b Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide

C99-006: A Randomized, Third-party Blind, Placebo-Controlled Pilot Study of the Effect of h5G1 .I-mAb on Dermatomyositis Patients

E99-001: Open-Label Study of the Long-Term Safety of Eculizumab (h5G1.1-mAb) in Rheumatoid Arthritis Patients Receiving Methotrexate who Previously Participated in Study C99-001

C99-001: A Phase 2 Randomized, Double-Blind, Placebo-Controlled Study of the Effect of Eculizumab (h5G1.1-mAb) on Rheumatoid Arthritis Patients Receiving Methotrexate

C97-002: Phase 1 Study of h5G1.1-mAb in Patients With Systemic Lupus Erythematosus

C97-001: Phase 1 Study of h5G1.1-mAb in Patients With Rheumatoid Arthritis

EMPLOYMENT HISTORY (cont.)

Name of Employer	Bayer Corporation, Metabolics & Rhuematic Research
Employer Address (City, State, Country)	West Haven, CT, USA
Dates of Employment	December 1996 to October 1998
Title	Medical Research Associate
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	October 1994 to December 1996
Title	Research Scientist
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	July 1990 to October 1994
Title	Senior Associate Research Scientist
Name of Employer	Bayer Corporation, Institute of Bone and Joint Disease & Cancer
Dates of Employment	December 1988 to July 1990
Title	Associate Research Scientist
Name of Employer	Genentech Incorporated, Immunology
Employer Address (City, State, Country)	South San Francisco, CA, USA
Dates of Employment	September 1986 to December 1988
Title	Research Assistant
Name of Employer	Genentech Incorporated, Bioassay Services
Dates of Employment	August 1984 to September 1986
Title	Laboratory Assistant

EDUCATION

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CURRICULUM VITAE

Employee Name	MICHAEL BOMBARA

Degree, Major	BS, Microbiology
Year Degree Conferred	1984
School	University of Maine
Address of School (City, State, Country)	Orono, Maine, USA

Signature: _____ **Date:** 26 Mar 2019

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Exhibit B

Study Name	Study Number	References	Explicit Confidentiality Provision in Clinical Study Protocol
An Open-Label Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Haemoglobinuria Who Previously Participated in Study C02-001 An Open-Label Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal Nocturnal Hemoglobinuria Who Previously Participated in Study E02-001	C02-001 E02-001 X03-001	D1, D2, D3, D4, D5	Yes
A Randomized, Double-Blind, Placebo-Controlled Pilot Study of the Effect of h5G1.1-mAb on Dermatomyositis Patients	C99-006	D4	Yes
Phase I Study of h5G1.1-mAb in Patients with Rheumatoid Arthritis	C97-001	D4	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis Patients Receiving Methotrexate	C99-001	D4	Yes
A Phase IIB Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on Rheumatoid Arthritis (RA) Patients with Active Disease Despite Receiving Methotrexate or Leflunomide	C01-004	D4	Yes
A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Effect of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy An Open Label Study of the Long-Term Safety of h5G1.1-mAb on the Reduction of Proteinuria in Patients with Idiopathic Membranous Glomerulopathy	C99-004 E99-004	D4	Yes
Phase I Study of h5G1.1-mAb in Patients with Systemic Lupus Erythematosus	C97-002	D4	Yes

Alexion Pharmaceuticals Inc.
352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

MASTER CLINICAL STUDY AGREEMENT

THIS AGREEMENT is made as of this 1st day of April, 2002 between The Leeds Teaching Hospitals NHS Trust located at Department of Haematology , Great George Street, Leeds LS1 3EX, UK ("Institution"), and Alexion Pharmaceuticals, Inc., a Delaware corporation, located at 352 Knotter Drive, Cheshire, CT 06410 ("Alexion");

WHEREAS, Alexion may from time to time desire Institution to conduct clinical studies (each, a "Study"), of one or more drug candidates (each, a "Study Drug"), pursuant to mutually agreed protocols which shall be incorporated herewith as Exhibit I (each, a "Protocol"), at Institution's facilities through the principal investigator (each, a "Principal Investigator") referred to on mutually agreed study specific exhibits incorporated herewith as Exhibit II (each, a "Study Specific Exhibit"); and

WHEREAS, by executing any Study Specific Agreement, Institution represents it is equipped to undertake the relevant Study, and Institution shall perform such Study on the terms and conditions as hereinafter set forth;

NOW, THEREFORE, in consideration of the mutual promises expressed herein, each bargained for and given in exchange for the other, the parties agree as follows:

1. **STUDY INITIATION:** Each Protocol will become effective when signed by Alexion and the relevant Principal Investigator. Each Study Specific Exhibit will become effective when signed by Alexion, the Institution and the relevant Principal Investigator.

2. **DATA AND INVENTIONS:** Institution agrees that all data, inventions, and discoveries generated during the course of a Study by Institution, any Principal Investigator or any employee or agent of Institution, or which otherwise relates to a Study Drug shall belong solely and exclusively to Alexion. Institution agrees to execute (and cause each such Principal Investigator, employee or agent to execute) any documents or undertake any further actions if requested by Alexion to evidence transfer of title thereto.

3. **SCOPE OF STUDY:** Institution agrees to exercise, and to cause each Principal Investigator to exercise his or her reasonable and diligent efforts and professional expertise to complete each Study in accordance with the relevant Protocol in a professional, efficient and timely manner. No other investigator may be substituted for the Principal Investigator on any Study, but the Principal Investigator may utilize the services of associates, including co-investigators, employed by the Institution as required; provided, however, that the Institution and Principal Investigator shall remain liable for the acts and omissions of such associates.

4. **TERM:** Subject to earlier termination pursuant to Section 6, the term of this

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Agreement shall commence on the date that it is made and continue until the later of (i) December 31, 2005 and (ii) the completion of any Study that remains ongoing as of December 31, 2005.

5. **FINANCIAL CONSIDERATION AND PAYMENT SCHEDULE:** Payment terms and schedules for each Study shall be included in each relevant Study Specific Exhibit.

6. **TERMINATION:** Any Study may be terminated by Alexion at any time in the exercise of its sole discretion upon fifteen (15) days prior written notice to Institution. Any Study may be terminated by Institution upon fifteen (15) days prior written notice to Alexion in the case of significant patient safety concerns. Upon receipt or giving of notice, as the case may be, Institution agrees promptly to terminate conduct of such Study to the extent medically permissible for all patients. In the event of termination of a Study hereunder, other than as a result of a material breach by Institution or the relevant Principal Investigator, the total sums payable by Alexion pursuant to this Agreement in respect of such Study shall be equitably prorated for actual work performed to the date of termination, with any unexpended funds previously paid by Alexion to Institution being refunded to Alexion. Either Institution or Alexion may terminate this Agreement upon written notice to the other at any time in which no Study is ongoing.

7. **CONFIDENTIALITY AND PUBLICATION:** Institution agrees, and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study 1) information that is disclosed by or on behalf of Alexion to Institution or the relevant Principal Investigator and 2) data, inventions, and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug. The preceding obligations shall not apply to information, data, inventions, or discoveries which have entered the public domain through no fault of Institution, or where Alexion agrees in writing to its use or disclosure or which was known by Institution prior to its date of disclosure by Alexion hereunder. Institution agrees to return confidential information of Alexion, and all copies thereof, at the conclusion of each Study or at an earlier date at the request of Alexion, except with respect to raw research Study data where original data will be retained if required by United States Food and Drug Administration regulations.

   Notwithstanding the foregoing, Principal Investigator may publish Study results, subject to the direction of Alexion's multi-center publication committee (if one has been constituted for the relevant Study) and provided a copy of any proposed verbal or written publication is received by Alexion at least 45 days in advance of submission for publication for review and comment on the appropriateness of the data analysis and presentation. If the parties disagree concerning the appropriateness of the data analysis and presentation, Institution and Principal Investigator agree to meet with Alexion representatives at the clinical study site or as otherwise agreed, prior to submission for publication, for the purpose of making good faith efforts to discuss and resolve any issues or disagreement. Alexion may

cause Principal Investigator to delay any such verbal or written publication for 60 days following its receipt if Alexion determines that such period is necessary or desirable to protect its rights to any intellectual property described therein. Furthermore, Alexion may cause Principal Investigator to remove any Alexion confidential information from such proposed verbal or written publication.

The provisions in this Section shall survive the termination or expiration of this Agreement.

8. **REPORTING OF DATA:** Institution agrees to provide Alexion periodically and in a timely manner during the term of this Agreement all the data called for in each Protocol on properly completed case reporting forms. Institution agrees to notify Alexion at the earliest time possible after learning of an adverse experience or drug reaction of any patient in any Study.

9. **COMPLIANCE WITH APPLICABLE LAWS:** Institution agrees to conduct each Study and maintain records and data during and after the term of this Agreement in compliance with all applicable legal and regulatory requirements, including without limitation, any applicable requirements of the United States Food and Drug Administration, including 21CRF Part 312 Subpart D.

Institution shall cause the relevant Principal Investigator to obtain informed consent forms from all subjects participating in each Study. The parties agree that Institution and/or Principal Investigator shall be responsible for the contents of the informed consent forms and shall have the right to modify such informed consent forms in a manner consistent with 21 CFR Part 50 Subpart B, as otherwise required by law and as requested by Institution's IRB. Institution and Principal Investigator agree not to include representations or obligations of Alexion in any informed consent forms.

10. **MONITORING OF STUDY:** During the term of this Agreement, Institution agrees to permit representatives of Alexion to examine at any reasonable time during normal business hours 1) the facilities where any Study is being conducted, 2) raw study data, and 3) any other relevant information (and to make copies) necessary for Alexion to confirm that each Study is being conducted in conformance with the relevant Protocol and in compliance with all laws and regulations, including those of the United States Food and Drug Administration. Original copies of each Study participant's individual medical records shall remain the property of Institution.

Alexion may contract with a third party to coordinate and monitor the conduct of all or part of any Study on behalf of Alexion, and Institution will cooperate with such third party to the same extent as it is obligated to cooperate with Alexion.

11. **PUBLICITY:** Neither party shall use the name of the other party or the name of the other party's affiliated companies, employees, or products for promotional purposes in connection with this Agreement without the prior written consent of the party

whose name is proposed to be used. Notwithstanding the foregoing, Alexion shall be permitted to state orally and in writing the fact that a Study is being, or has been, conducted at Institution under the direction of the relevant Principal Investigator.

12. **INDEPENDENT CONTRACTOR:** Institution and each Principal Investigator is acting in the capacity of an independent contractor hereunder and not as an employee or agent of Alexion.

13. **CONTROLLING LAW:** This Agreement shall be governed by and construed in accordance with English law (other than provisions relating to conflicts of laws).

14. **AGREEMENT MODIFICATIONS:** This Agreement may not be altered, amended, or modified except by a written document signed by all parties.

15. **INCONSISTENCIES:** In the event of any conflict or inconsistency between the terms and conditions of this Agreement and the terms and conditions of any Protocol, Study Specific Exhibit or other documents relating to any Study, the terms and conditions of this Agreement shall govern.

16. **NOTICE:** All notices given hereunder shall be in writing and shall be delivered by hand, facsimile, certified or registered mail, or overnight delivery by a nationally recognized carrier, addressed to the parties as follows:

To: Alexion: Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Attn: Clinical Accounts Associate
Facsimile #: 203-271-8196

Copy To: Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Attn: Vice President and General Counsel
Facsimile #: 203-271-8198

To: Institution: The Leeds Teaching Hospitals NHS Trust
Department of Haematology
Great George Street
Leeds LS1 3EX, UK

Notice shall be deemed given when received and the parties may change the respective addresses where notice is to be given upon prior notice hereunder.

17. **INDEMNITY:** Alexion agrees to indemnify, hold harmless, and defend Institution, Principal Investigator, co-investigator(s), its trustees, regents, directors, officers,

employees, physicians, and all other qualified personnel working under their supervision (collectively, the "Indemnitees") from and against all claims, demands, actions, and proceedings (collectively, "Claims") which may be brought or asserted against Indemnitees to recover damages and losses for or attributable to bodily injury, sickness, disease, or death arising directly and solely out of any Study.

Alexion does not, however, agree to indemnify, hold harmless, or defend Indemnitees from Claims resulting from:

a. failure of any Indemnitee to evaluate or properly interpret available information that is relevant to any Study and for independent decisions made as a result of such failure;

b. failure of any Indemnitee to adhere to all provisions of this Agreement and the Protocol for any Study or to written recommendations and written instructions delivered to such Indemnitee by or on behalf of Alexion concerning the administration and use of any drug substances, including placebo, involved in such Study; or

c. failure of any Indemnitee to render professional service or to conduct any Study in a normal, prudent manner.

A condition of this indemnity obligation is that, whenever an Indemnitee has information from which it may reasonably conclude an incident of bodily injury, sickness, disease, or death is likely to occur or has occurred, such Indemnitee shall immediately give notice to Alexion of all pertinent data surrounding such incident, and in the event a Claim is brought or asserted, such Indemnitee shall assist Alexion and cooperate in the defense thereof, including by gathering information with respect to the time, place, and circumstances and in obtaining the names and addresses of the injured parties and available witnesses. No Indemnitee may, except at its own cost, voluntarily make any payment or incur any expense in connection with any Claim without the prior written consent of Alexion.

The Institution and Principal Investigator shall maintain professional liability insurance as shall be reasonably necessary to insure themselves against any claims for damages whether arising by reason of loss of property, personal injuries or death occasioned in connection with the Study. The Institution and Principal Investigator shall provide a Certificate of Insurance evidencing such coverage to Alexion upon request.

Subject to its internal policies regarding self-insurance, Alexion shall maintain such comprehensive general liability and products liability insurance as shall be reasonably necessary to insure itself against claims for damages arising out of the Study. Alexion shall provide a Certificate of Insurance evidencing such coverage to the Institution upon request.

Institution will ensure that the Study is approved by the Trust Management and that all Managerial and Ethical approval has been obtained, and accordingly that all medical and other staff participating in the Study will be covered by NHS indemnity.

18. **GENERIC DRUG ENFORCEMENT ACT OF 1992:** Institution represents that it and its employees, affiliates, and agents working on each Study have never been 1) debarred or 2) convicted of a crime for which a person can be debarred, under Section 306(a) or 306(b) of the Generic Drug Enforcement Act of 1992 ("Section 306(a) or (b)"). Institution represents that it has never been and, to the best of its knowledge after due inquiry, none of its employees, affiliates, or agents working on each Study has ever been 1) threatened to be debarred or 2) indicted for a crime or otherwise engaged in conduct for which a person can be debarred, under Section 306(a) or (b). Institution agrees that it will promptly notify Alexion in the event of any such debarment, conviction, threat, or indictment. The terms of the preceding sentence shall survive the termination or expiration of this Agreement.

19. **FINANCIAL DISCLOSURE BY INVESTIGATORS**: Institution shall cause each Principal Investigator and each other investigator or sub-investigator, if any, listed in the Form 1572 or identified in the Protocol and directly involved in the treatment or evaluation of research subjects in connection with any Study to complete and deliver to Alexion a financial disclosure questionnaire sufficient to allow Alexion to comply with 21 CFR Part 54. Institution shall cause such persons to respond promptly to any requests by Alexion for additional information or supporting documentation relating to the responses on such financial disclosure questionnaire(s).

IN WITNESS WHEREOF, the parties hereto have caused this Agreement to be executed by their duly authorized representatives as of the date first above written.

ALEXION PHARMACEUTICALS, INC.

By: _____
Name: Thomas I. H. Dubin
Title: Vice President and General Counsel

The Leeds Teaching Hospitals NHS Trust

DR. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

By: _____
Name: Dr. D.R. Norfolk.
Title: Associate R&D Director
30/4/02

EXHIBIT I
PROTOCOL
(SUBJECT TO CHANGE WITH EACH STUDY)

EXHIBIT II
STUDY SPECIFIC EXHIBIT
(SUBJECT TO CHANGE WITH EACH STUDY)

Alexion Pharmaceuticals Inc.
352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

Alexion Pharmaceuticals, Inc.
EXHIBIT II, ATTACHED TO MASTER CLINICAL STUDY AGREEMENT, FOR SPECIFIC STUDY SCOPE, BUDGET & PAYMENT SCHEDULE
with The Leeds Teaching Hospitals NHS Trust

Study Specifications

Upon signature, this Exhibit will be incorporated into and made part of the Master Clinical Study Agreement between Alexion Pharmaceuticals, Inc. ("Alexion") and The Leeds Teaching Hospitals NHS Trust ("Institution") dated, April 1, 2002

1. Product/Compound: h5G1.1-mAb ("Study Drug")

2. Protocol # and Title: C02-001, entitled: "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria" ("Study").

3. Principal Investigator: Peter Hillman, MD

4. Contract Research Organization (if applicable, if not put N/A): Kendle

5. Scope:

   The Study will begin following IRB approval and receipt by Alexion of relevant Study documents. The Study will begin around May 1, 2002 and the anticipated completion date is December 31, 2003. Such dates may be modified by written agreement executed by Principal Investigator and authorized representatives of both Institution and Alexion.

6. Subjects:

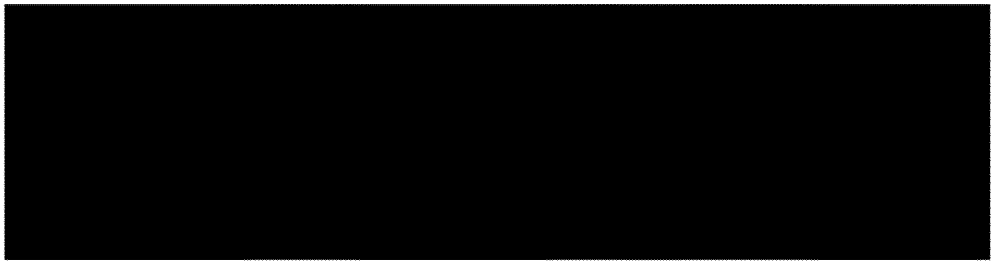
   The anticipated number of completed and valid subjects in the Study shall be enrolled within the time frame described above, and according to the criteria listed in the Protocol.

[redacted]









7.2 Definitions:

A "Qualified Study Subject" is a research subject who has been randomized and has met the entrance criteria as defined in the Protocol or who is given written permission by Alexion to enter the Study.

A "Protocol Compliant Study Subject" is a Qualified Study Subject who has met the minimum attendance and compliance standards in the Protocol for evaluation of effectiveness of the Study Drug, and has completed the Study.

A "Non-Protocol Compliant Study Subject" is a Qualified Study Subject that violates the Study procedures, such as, insufficient clinic attendance, poor protocol compliance or other protocol violations, as defined in the Protocol, but the subject completes the Study.

An "Early-Terminated Study Subject" is a Qualified Study Subject who has been withdrawn from the Study because of: lack of efficacy, adverse event, lost to follow-up, or voluntary patient withdrawal. Additional follow-up may be required for these patients to meet FDA regulations.

A "Screen Failure Study Subject" is a study subject who meets all entrance criteria and consents to enroll in the study but fails to randomize due to, e.g., unacceptable laboratory values at the screening visit.

A "Non-Qualified Study Subject" is a research subject who has not met the entrance criteria as defined in the Protocol.



[redacted]

[redacted]

8. Provisions of Master Clinical Study Agreement

The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated, April 1, 2002. Without limiting the foregoing, the Principal Investigator agrees to the intellectual property provisions of Section 2 of the Master Clinical Study Agreement and the confidentiality provisions of Section 7 of the Master Clinical Study Agreement.

9. Notices

All communications and notices required or permitted under this Agreement shall be deemed sufficiently served if hand delivered or served by certified mail, postage prepaid, addressed as follows:

TO ALEXION: Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO PRINCIPAL INVESTIGATOR:
Peter Hillman, MD
Algernon Firth Building
Leeds General Infirmary
Great George Street
Leeds LS1 3EX

TO INSTITUTION: The Leeds Teaching Hospitals NHS Trust
Department of Haematology
Great George Street
Leeds LS1 3EX

ACCEPTED BY:

Alexion Pharmaceuticals, Inc.

By: _____
Thomas I. H. Dubin
Vice President and General Counsel

Date: 5-2-02

The Leeds Teaching Hospitals NHS Trust

By: _____
Dr. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

Date: 18/06/02

The undersigned, as Principal Investigator, hereby agrees to accept responsibility for performance of the Protocol described herein, acknowledges having reviewed this Agreement and agrees to be bound by the terms and conditions stated herein and in the Master Clinical Study Agreement dated, April 1, 2002, which is incorporated herein by reference.

PRINCIPAL INVESTIGATOR

_____
Peter Hillman, MD

10/JUNE/02
Date

Attachment A
Study Budget

Working Protocol

CONFIDENTIAL

IND NUMBER:	Pending
STUDY NUMBER:	C02-001
VERSION:	2.1
USAN:	ANTI-C5 ANTIBODY
DOSAGE FORM:	PARENTERAL
STUDY TITLE:	AN OPEN-LABEL STUDY OF THE EFFECT OF ECULIZUMAB (h5G1.1-mAb) ON PATIENTS WITH TRANSFUSION DEPENDENT, HEMOLYTIC, PAROXYSMAL NOCTURNAL HEMOGLOBINURIA
CLINICAL DIRECTOR:	CHRISTOPHER F. MOJCIK, M.D., Ph.D.
ADDRESS:	ALEXION PHARMACEUTICALS, INC. 352 KNOTTER DRIVE CHESHIRE, CT 06410
TELEPHONE NUMBER:	203-272-2596 (OFFICE) 203-784-4333 (EMERGENCIES)
PROJECT SPONSOR:	ALEXION PHARMACEUTICALS, INC.
DATE:	MARCH 15, 2002

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

Principal Investigator — Date

Christopher F. Mojcik, MD, PhD — Date 3/15/02

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FIGURE 1

STUDY FLOW CHART

Visit	1	2	3	4	5	6	7	8	9	10	11	12	PT
Day (Month)	-14 to -21	0	7	14	21	28 (1)	35	42	56 (2)	70	84 (3)	112 (4)	
Assessment	SCR	Baseline											
Dosing (mg)		600	600	600	600	900		900	900	900	(900)*	(900)*	
Informed consent	x										(x)*		
Medical history**	x												
Weight	x										(x)*		
Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x										x		x
12-lead ECG	x										x		x
Hematology	x	x	x	x	x	x	x	x	x	x	x	(x)*	x
Blood chemistry	x	x	x	x	x	x	x	x	x	x	x	(x)*	x
Ferritin, Vitamin B12, Folate	x										x		x
Coagulation Profile		x	x	x	x	x			x		x		x
Urinalysis	x	x				x			x		x	(x)*	x
Reticulocyte count		x	x	x	x	x	x	x	x	x	x		x
Haptoglobin		x		x		x		x	x	x	x		x
Plasma (free) hemoglobin		x									x		x
Ham Test		x									x		x
Flow cytometry	x	x	x	x	x	x	x	x	x	x	x		x
EORTC QLQ-C30		x				x			x		x		x
Self assessment urine diary		x	x	x	x	x	x	x	x	x	x		x
Neisserial throat culture	x	x				x			x		x		x
PK/PD/SC5b-9		t/p			t/p	t/p	i	t		t/p	t		t
Anti-h5G1.1-mAb (HAHA)		x				x			x		x		x
Concomitant Medications	x	x	x	x	x	x	x	x	x	x	x	x	x
Adverse events		x	x	x	x	x	x	x	x	x	x	x	x
Hepatitis Screen	x												
Pregnancy (β-HCG)	x										x		x
Neisserial vaccine	x												
Exchange transfusion											x***		x^

Abbreviations: PT = Premature Termination; SCR = Screening; PK = Pharmacokinetics; PD = Pharmacodynamics; t = trough sample; p = peak (1 hour after dosing) sample; i = interval sample. * = Only if participating in extension study. * = Includes laboratory values from the 3 months prior to screening. ***Only if not participating in extension study and PNH RBC's >50% of total RBC's by flow cytometry. ^ Only if PNH RBC's >50% of total RBC's by flow cytometry.

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GLOSSARY OF ABBREVIATIONS
(in alphabetical order)

BUN	=	blood urea nitrogen
CRF	=	case report form
ECG	=	electrocardiogram
GCP	=	good clinical practice
Hct	=	hematocrit
Hgb	=	hemoglobin
IRB	=	institutional review board
LDH	=	lactic dehydrogenase
mmHg	=	millimeters of mercury
OTC	=	over-the-counter
Plt	=	platelet
PMN	=	polymorphonuclear leucocyte (neutrophil)
PNH	=	paroxysmal nocturnal hemoglobinuria
PRBC	=	packed red blood cells
RBC	=	red blood cell
sec	=	seconds
SGOT	=	serum glutamic-oxaloacetic transaminase (AST; aspartate aminotransferase)
SGPT	=	serum glutamic-pyruvic transaminase (ALT; alanine aminotransferase)
ULN	=	upper limit of normal

NOTE ON USAGE OF TERMS:

- The term "Investigator" refers to the principal investigator or responsible physician.
- The term "study staff" refers to the Investigator or associates of the Investigator.
- The term "Pharmacist" refers to the staff member responsible for preparing study medication for dispensing.

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STUDY SYNOPSIS

COMPOUND	h5G1.1-mAb
STUDY TITLE	An Open-Label Study of The Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxysmal Nocturnal Hemoglobinuria.
DEV'T PHASE	Pilot
KEY TARGET DATES	Study Start: May 1, 2002 Last Subject Enrolled: July 31, 2002 Last Subject Completed: November 30, 2002
INDICATION	Subjects to be recruited for this study will be patients with transfusion dependent, hemolytic, PNH.
OBJECTIVES	The primary objective is to determine the safety and tolerability of h5G1.1-mAb and to evaluate the pharmacokinetics and pharmacodynamics of this antibody in the target population.
DESIGN	Open-label.
SUBJECTS	Subjects to be recruited for this study will be patients with transfusion dependent PNH.
SAMPLE SIZE	10 subjects
TREATMENTS	Eculizumab (h5G1.1-mAb) 600 mg via IV bolus over 30 minutes every week for 4 doses, then 900 mg via IV bolus over 30 minutes every other week for 4 doses.
CLINICAL EFFICACY AND SAFETY VARIABLES	Efficacy Variables: Primary-none designated, as this is a pilot study. Secondary-number of units PRBCs transfused; time to first transfusion of PRBCs; change from baseline hemoglobin and hematocrit; change from baseline indicators of hemolysis (reticulocyte count, haptoglobin, LDH and bilirubin); number of paroxysms and time to first paroxysm; reticulocyte count; haptoglobin; change from baseline EORTC QLQ-C30; pharmaco-dynamics. Exploratory-platelet and neutrophil count; flow cytometry; Ham test; plasma (free) hemoglobin Safety Variables: Adverse events, laboratory data, ECGs.
PROCEDURE	Enrollment occurs following satisfaction of entry criteria. Subjects receive study medication as a constant infusion over 30 minutes: 600 mg weekly for 4 weeks and then 900 mg every other week for 6 weeks, for a total of 8 treatments. Study procedures will be followed according to the flow sheet.

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NUMBER OF CENTERS	There will be up to 2 study centers in the United Kingdom.
DURATION OF STUDY PER SUBJECT	16 weeks.
STATISTICAL METHODS	**Efficacy Analysis** Mean change from baseline to post-baseline assessments for the secondary and exploratory endpoints will be summarized by the mean, median, standard deviation and range at each post-baseline assessment for each treatment group and overall. **Safety Analysis** The safety analysis will compare treatment groups with regard to adverse events, laboratory abnormalities and vital signs. Summary statistics will describe each category for laboratory abnormality and vital signs. Adverse events will be tabulated. Analysis of variance will test the difference between treatment groups and either CMH or Fisher's exact test will be employed for categorical data.
SPONSOR	ALEXION Pharmaceuticals, Inc. 352 Knotter Drive Cheshire, CT 06410
CLINICAL RESEARCH ORGANIZATION (CRO)	None.

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1. INTRODUCTION

PNH was first described in 1882 [1], and is an acquired clonal disorder of the hemopoietic stem cell. It is well known for its triad of clinical manifestations: intravascular hemolysis, thrombosis and reduced hematopoiesis [1, 2]. It is an uncommon condition with an incidence of somewhere between 1:100,000 and 1:1,000,000. The disease can present at almost any age (median of approximately 35 years) and with a roughly equal sex distribution [2-5].

The three cardinal features of PNH are hemolysis, cytopenias and thrombosis. There is a continuum from mainly hemolytic in type to predominantly aplastic with a subclinical PNH clone. The clinical characteristics, as well as clone size, may change throughout the course of the disease.

In a third of cases, hemolysis is the predominant symptom. The vast majority of hemolytic PNH patients (>80%) have prominent hemoglobinuria at some point in the disease [2], and a quarter of patients present with dark urine in the early morning. This is usually episodic and precipitated by infection, drugs or trauma (presumably related to complement activation). Hemoglobinuria very occasionally progresses to acute renal failure, which is usually reversible. There may also be esophageal spasm and erectile dysfunction [1], both symptoms of smooth muscle dysfunction, possibly caused by the adsorption of nitric oxide by free plasma hemoglobin.

Cytopenias may affect all lineages of hematopoiesis. Anemia may be associated with iron or folate deficiency as well as evidence of hemolysis. On bone marrow examination, erythroid hyperplasia is usually accompanied by hypoplasias and a variable degree of dyserythropoiesis. Further, there is an association of PNH with aplastic anemia and about one-third of patients have aplastic anemia diagnosed before the onset of PNH [2]. Thrombocytopenia is present in 80% of patients [2]. PNH granulocytes have normal lifespans [1], and unless PNH patients are significantly neutropenic, infections do not occur at an increased rate. This suggests that the membrane abnormalities do not significantly affect neutrophil function. Involvement of lymphocytes is usually minimal and not associated with immunodeficiency.

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Venous thrombosis in PNH occurs in a large minority (39%) of patients, and may be repetitive [2]. It tends to occur in unusual anatomic locations, such as hepatic veins [1]. Further, it often occurs without warning, and is a common cause of mortality for these patients [2, 5].

Flow cytometry is increasingly used for diagnosis and monitoring. Red cells and granulocytes display complete (type III) or partial (type II) deficiency of GPI linked proteins [6]. Type III cells are most sensitive to the effects of complement. (See below for a discussion of GPI-linked proteins and complement.) Patients often show the presence of Type II and Type III cells simultaneously [5]. The proportion of clonal subtypes (e.g. Type II or III cells) tends to predict the clinical phenotype [5]. On the whole, hemolytic patients have large clones (>50% PNH neutrophils) and hypoplastic patients smaller ones (<20% PNH neutrophils and <10% PNH erythrocytes)[7]. It seems that thrombosis, in particular, is related to larger clone size. A recent UK study found that the 10 year cumulative incidence rate of thrombosis was 39% in patients with granulocyte clone sizes of ≥50% but only 7% in those with clone sizes of <50% [8].

Although approximately 15% of PNH will spontaneously remit [2], the prognosis of PNH is generally quite poor. In the series by Dacie and Lewis [9], the median survival after diagnosis was found to be 8 years and in a recent review of the series this was increased to 10 years [2]. In the large retrospective multicenter study in France with 220 PNH patients between 1950 and 1995, the Kaplan Meier survival estimate at 10 years was 65% and at 15 years 48% [4]. 30% of these patients had a previous history of aplastic anemia. PNH is directly implicated in the cause of death in approximately a half of patients. The majority of deaths are due to venous thrombosis, with progressive cytopenias the second most common cause [1, 5].

Therapy for PNH is primarily symptomatic. Anemia prophylaxis with iron and folic acid can be effective, and transfusion should be avoided if possible. A potential problem with iron supplementation is that the resultant reticulocytosis may precipitate hemoglobinuria. Steroids are thought by some to reduce the frequency of attacks but there appears no reason that they should affect either the activation of complement or the proportion of PNH red cells. In one small

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series of five refractory and transfusion dependent patients, four benefited from Danazol and became transfusion independent [10]. Oral anticoagulation is used for prophylaxis of thrombosis, and thrombolytic therapy for acute thrombotic events. Immunosuppression with either anti-thymocyte globulin or Cyclosporine is effective in the 10% of patients with progressive pancytopenia [1, 11]. The only curative treatment is bone marrow transplantation, but due to its substantial morbidity and mortality, this procedure is available to only a small proportion of patients [1, 2].

The biochemical defect in PNH occurs in the synthesis of the glycosyl-phosphatidylinositol (GPI) anchor. This glycolipid structure is the means by which many proteins are attached to the plasma membrane. The *pig-a* (phosphatidyl-inositol glycan complementation class A) gene comprises 6 exons and spans 17 kilobases on the short arm of the X chromosome, at Xp22.1 [1]. PIG-A forms a complex with PIG-H, PIG-C and GPI1 [12] to mediate the first step in the biosynthesis of the GPI anchor. All reported patients with PNH have a haematopoietic GPI-deficient clone due to a somatic mutation of *pig-a* [5]. In fact, many patients have more than one independent GPI deficient clone with up to four being described in a single patient. Constitutional *pig-a* defects have not been described. A single mutation event is sufficient to cause disease because the *pig-a* gene is X-linked.

Unanchored membrane proteins are degraded in the endoplasmic reticulum or expelled from the cell. All GPI linked proteins have been found to be missing from PNH haemopoietic cells. GPI linked proteins have extremely diverse functional roles, including coreceptors, signal transduction, membrane transport, complement receptors, and complement regulation [1]. The two missing proteins thought to cause the clinical manifestations of the disease are DAF (Decay Accelerating Factor; CD55) and MIRL (membrane inhibitor of reactive lysis; CD59). DAF is an inhibitor of C3 and C5 convertases, accounting for the increased deposition of C3b on PNH cells after complement activation . However, patients with the Inab phenotype, in which CD55 is congenitally absent, have only slightly increased sensitivity to complement. It is therefore unlikely that absence of DAF is solely responsible for the intravascular hemolysis of PNH. MIRL is an inhibitor of membrane attack complexes. It binds to the C8 component of complement, preventing the binding and subsequent

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polymerization of C9. In the absence of MIRL, polymeric C9 is able to disrupt the cell membrane and this accounts for the intravascular hemolysis and possibly the thrombosis which occur in PNH. The major role of MIRL is supported by the fact that congenital absence of CD59 is associated with clinical hemolysis, hemoglobinuria and venous thrombosis.

There is an illustrative case report of a woman with C9 (complement component 9) deficiency and PNH. However, her clinical course was clearly distinctive for PNH. Her RBC's were approximately 80% Type III and 95% DAF negative. Despite this, she suffered only mild hemolysis until she received a postoperative blood transfusion (which reversed her C9 deficiency). She then had episodes of gross hemoglobinuria and jaundice, indicative of spontaneous, massive hemolysis [13].

The proinflammatory activities of the activated terminal complement components C5a and C5b-9 have been implicated in a wide range of inflammatory disease states [14]. The complement system is comprised of over thirty serum and cell surface proteins that subserve important roles in immune recognition and effector function [15]. Upstream activation of complement through either the classical or alternative pathways leads to the cleavage of C3 into C3a, a weak anaphylatoxin, and C3b, which is critical for opsonization of pathogenic microorganisms and for the clearance of immune complexes. C3b also participates in the activation of C5, which results in the production of C5a and C5b. C5a is a potent anaphylatoxin, chemotactic factor, and cell activating molecule that mediate multiple proinflammatory activities [16]. C5b recruits the terminal complement components C6-9 to form the terminal complement complex, C5b-9. This complex is cytolytic when present in large quantities, or proinflammatory when present in sublytic quantities. It is capable of disrupting normal homeostasis following deposition on platelets and endothelial cells [14, 17].

Eculizumab (h5G1.1-mAb) is a humanized monoclonal antibody to human C5. It binds to C5 and prevents its cleavage into C5a and C5b. It has been used in single dose Phase I studies in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients. In a dose escalation study of 42 RA patients, h5G1.1-mAb was safe and well tolerated up to 8 mg/kg. There was one serious adverse event (SAE) in the lowest dose cohort, in which a single patient had 2

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flares of his RA, which required hospitalization for treatment with high-dose steroids and plasmapheresis. This SAE was not considered related to study medication by the principal investigator (PI). Otherwise, there were no dose-dependent trends in adverse events noted across dose groups, and there were no clinically significant laboratory abnormalities noted. Serum hemolytic activity (a surrogate of complement activity) was completely blocked for an average of 7 days at the 8 mg/kg dose level (n=6) and did not return to baseline levels until day 14. (Data available on file at Alexion Pharmaceuticals, Inc.)

In 24 SLE patients, a similar dose escalation study was performed. Again, h5G1.1-mAb was safe and well tolerated up to 8 mg/kg. There were three SAEs in this study: 1) a patient in the placebo group suffered a fractured ankle; 2) a patient in the 4 mg/kg dose group had a near-syncopal episode; and 3) a patient in the 8 mg/kg dose group had an exacerbation of asthma. None of these SAEs were considered related to study medication by the PI's involved. Otherwise, there were no dose-dependent trends in adverse events noted across dose groups, and there were no clinically significant laboratory abnormalities noted. Serum hemolytic activity blockade was similar to that seen in the RA study. (Data available on file at Alexion Pharmaceuticals, Inc.)

A Phase IIa, multi-center, double blind, placebo-controlled trial was then performed, testing three dosages of h5G1.1-mAb in patients with rheumatoid arthritis also taking methotrexate. Treatment arms included: 1) placebo (PLC); 2) h5G1.1-mAb 8mg/kg q 2 weeks (Q2wk); 3) h5G1.1-mAb 8mg/kg q week X5, then q month (Induction/Q4wks); 4) h5G1.1-mAb 8mg/kg q week X5, then q 2 weeks (Induction/Q2wks). Thus, the maximum cumulative dose given was 72 mg/kg. There were 209 patients treated for 3 months, then followed for 3 additional months off study medication. The agent showed an inverted U-shaped dose-response curve, with the Induction/Q4 wks regimen showing moderate efficacy (44% ACR 20 vs. 18% placebo rate) at 3 months. In a subpopulation of patients with elevated baseline serum C5b-9 levels, a larger percentage of patients in the active treatment groups responded, and the agent displayed a more conventional dose-response curve. Following the withdrawal of this agent, patients tended to worsen in the majority of clinical measures. There were no safety concerns noted with any of the regimens used. (Data available on file at Alexion Pharmaceuticals, Inc.) Further, this trial was overseen by a data safety

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and monitoring board (DSMB), which periodically reviewed safety data. There were no concerns raised by this panel regarding the trial.

This agent is also being used in a Phase II study of idiopathic membranous nephropathy patients. In this study, patients are treated with Eculizumab for 4 months at dosages of 8 mg/kg every 2 weeks or every 4 weeks, or placebo. The maximum cumulative dose given was also 72 mg/kg. This trial is still blinded, so results are not currently available; however, there appear to be no safety issues to date. As with the RA trial noted above, the membranous trial is monitored by a DSMB. They have raised no safety concerns thus far.

For both the Phase IIa RA study, and the Phase II membranous study, year long extension studies have been offered. One hundred and thirty-two RA patients entered the extension (receiving 8 mg/kg every 2 weeks); to date, 70 patients have completed a year of therapy. Thus, there are over 107 patient-years of exposure in this population. In the membranous group, 60 patients have enrolled; thus far 21 have completed a year. There are over 40 patient-years of exposure in this population.

Across all trials thus far, there has been one incident of meningococcal meningitis. This occurred in a membranous nephropathy patient who had completed the blinded Phase II study and had been on open label (extension study) therapy for 3 months. She recovered with antibiotic therapy and was left with necrosis of several digit tips as sequelae (see section 7, below).

A related compound is h5G1.1-scFv, which has been used in a 24 hour regimen in over 3000 patients in cardiovascular intervention trials. This agent was shown to potently inhibit the generation of activated terminal complement byproducts such as C5b-9, but did not diminish the generation of C3b [18].

If additional information is needed, please refer to the Investigator's Brochure.

2. STUDY OBJECTIVES

The objectives of this study are:

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1. To determine the clinical and laboratory safety and tolerability of Eculizumab administered intravenously (IV) in patients with PNH;
2. To obtain information on the pharmacokinetics and pharmacodynamics of this antibody in the target population.

3. OVERALL DESIGN AND PLAN OF STUDY

This is an Open-Label Pilot study of Eculizumab administered to patients with transfusion dependent, hemolytic, PNH.

There will be 10 patients treated. Following a screening period of between 14 and 21 days, a 10 week, open-label treatment period will commence. Patients will receive Eculizumab (h5G1.1-mAb, 600 mg) by bolus infusion every week for 4 weeks, then Eculizumab (900 mg) by bolus infusion every other week for 6 weeks. All patients will receive 8 infusions of Eculizumab.

Patient safety will be assessed via the collection of adverse events and the evaluation of pre- and post-treatment laboratory data. In addition, various efficacy parameters, e.g., transfusion requirements, blood indices, and EORTC QLQ-C30, will be evaluated.

4. ETHICS AND GOOD CLINICAL PRACTICE

- This study will be conducted in accordance with Alexion standard operating procedures, which are designed to ensure adherence to Good Clinical Practice (GCP) as required by the following:
- Directive 91/507/EEC: The Rules Governing Medicinal Products in the European Community;
- Code of Federal Regulations dealing with clinical studies (21 CFR including parts 50 and 56 concerning informed consent and IRB regulations); and
- Declaration of Helsinki, concerning medical research in humans ("Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects," Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996, and Edinburgh, 2000).

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- Food and Drug Regulations dealing with clinical studies (Part C) and associated Guidance for Clinical Trial Sponsors (draft 2001/08/01)
- Note for Guidance on Good Clinical Practice in valid version approved by CPMP in July 1996.
- International Conference on Harmonization; Good Clinical Practice: Consolidated Guideline; Notice of Availability, in Federal Register, May 9, 1997; adopted by Therapeutic Products Directorate of Health Canada, 1997.
- Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, Medical Research Council of Canada, Social Sciences and Humanities Research Council of Canada, August 1998

5. STUDY POPULATION

5.1 Primary Diagnosis

The study population will comprise individuals who have transfusion dependent, hemolytic, PNH.

Patients will be treated in accordance with the standard medical practice of the physician charged with their care, with few exceptions: please see Section 6.7, Concomitant Treatment, below.

5.2 Enrollment Criteria

- Age $\geq$ 18 years.
- Transfusion dependent (i.e. at least 4 transfusions in the last 12 months) hemolytic PNH.
- Presence of a GPI deficient clone.
- Disease duration $\geq$ 6 months.
- No new immunosuppressive agents x 84 days prior to first dose of study medication.
- No history of bone marrow transplantation.
- Patient must be willing and able to give written Informed Consent.

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- Patients must be post-menopausal, surgically sterile or practicing a medically approved method of contraception.
- A negative throat culture for N. meningitidis or N. gonorrhoeae. If the screening throat culture comes back positive for these species, the patient must be treated with appropriate antibiotics, and may be re-tested. If the repeat throat culture is negative for these organisms, the patient is then eligible. Study centers must wait for the final throat culture report from the central lab before making the decision as to a patient's eligibility for the study. Patients that test positive for non-pathogenic species of Neisseria may be enrolled as long as there is no history of meningococcal disease or symptoms of active infection.

5.3 Exclusion Criteria

- Liver disease, defined as AST/ALT and bilirubin $\geq$ 1.8 times the upper limit of normal (ULN), not related to PNH, at screening.
- History of alcohol or drug abuse within two years of screening.
- A history of positive Hep B or Hep C serology.
- Renal insufficiency, defined as serum creatinine $\geq$ 2 X ULN.
- History of malignancy, excepting non-melanoma skin cancer and remote ($\geq$ 2 years) malignancies in complete remission.
- Presence or suspicion of active bacterial infection in the opinion of the investigator, or recurrent bacterial infections.
- Known or suspected hereditary complement deficiency.
- Participation in any other investigational drug trial or exposure to other investigational agent, device, or procedure within 30 days prior to screening.
- Pregnant or breast-feeding patients and women intending to conceive during the course of the study, including the follow-up period.
- History of meningococcal disease.
- Poorly controlled diabetes, defined as blood glucose greater than 300 mg/dl, or recent (within 3 months) HbA1c > 10.5%.
- History of allergic reaction to murine proteins.
- Prior exposure to h5G1.1-mAb.

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- Any clinically significant medical condition that, in the opinion of the investigator, is likely to interfere with the patient's participation in the study or the evaluation of the study medication's safety profile.
- Patients who are not vaccinated against N. meningitidis

5.4 Number of Patients

It is estimated that 10 patients will be treated. There will be up to 2 study centers.

5.5 Method of Patient Assignment and Blinding Procedure

None, as this is an open-label study. All patients will receive Eculizumab.

6. DRUGS, DOSAGE AND DURATION OF TREATMENT

6.1 Test Material and Control Agents

Eculizumab (h5G1.1-mAb) 10 mg/ml

6.2 Supply, Packaging, Labeling and Storage

The active study medication is manufactured by Alexion Pharmaceuticals, Inc., and is shipped to Clinical Trial Services, who will supply it to the sites. Each site will supply the appropriate diluent for mixing the study medication.

Eculizumab will be packaged as a solution for injection in 30 ml vials with a concentration of 10 mg/ml. It will be packaged in bulk containers and shipped to the site prior to study start. This bulk shipment will include specific directions as to the dilution at which the infusion will be administered.

Each vial of Eculizumab will have a two-part label describing the vial contents and a place for the pharmacist to record patient number, initials, and treatment number. The tear-off portion of the label is to be affixed to the label sheet provided.

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The site should notify Clinical Trial Services immediately if vials are damaged and cannot be used: Clinical Trial Services will ship additional drug at once. As with all investigational drugs, Eculizumab must be stored in a secure, limited access storage area. When study medication arrives, it should be promptly removed from the shipping cooler and stored in the refrigerator at 2° – 8°C.

6.3 Dosage and Administration

Preliminary pharmacokinetic analyses have been performed using data obtained from Phase II trials in rheumatoid arthritis and psoriasis completed by Alexion wherein patients were given 8.0mg/kg infusions of eculizumab. These analyses compared the actual attained steady-state serum concentrations at a trough and a mid interval between troughs with theoretical concentrations attained with a 600 mg fixed dose calculated from the same individuals. These data demonstrated that the 600mg fixed dose had favorable characteristics compared to dosing on a mg/kg basis. The geometric and arithmetic means for the trough concentrations were very similar (i.e. within 5 μg/ml of each other).

These preliminary analyses support the use of a fixed dose in this setting. There is little potential to overdose with either dosing system because there is no dose-related toxicity associated with this agent noted after treating more than 300 patients. Nevertheless, the fixed dose may produce lower peak values. A more likely problem therefore is the potential to underdose. However the use of a fixed dose of 600 mg appears to be at least at good as the weight based dosing system in obtaining trough concentrations in excess of 30 ug/ml.

Analysis of the pharmacodynamic properties of eculizumab in the Phase II study in Rheumatoid Arthritis patients revealed that serum complement activity was not completely blocked for the entire 12 week treatment period. In this study, the patients in the highest dose group received 8.0mg/kg for 5 weeks followed by biweekly infusions of 8.0mg/kg for 2 months. Serum complement activity was essentially completely blocked in all patients during the weekly dosing period but

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several patients exhibited a modest return of complement levels during the biweekly dosing schedule.

Because the primary lesion in PNH patients results from the lysis of susceptible erythrocytes by serum complement, we propose a modified dosing schedule for this study that should provide more complete blockade of serum complement during the proposed clinical study period. This modified regimen will employ the fixed weekly 600mg dosing schedule (as described above) for 4 weeks followed by a fixed 900mg biweekly dose for 2 months. This modified dosing regimen should increase eculizumab serum levels such that the PNH patient's serum complement levels are more completely blocked during the clinical study period.

Patients will receive 600 mg of Eculizumab every week X 4 doses, then 900 mg of Eculizumab every other week X 4 doses. See the Study Flow Chart on page 4.

All patients will receive 8 infusions of Eculizumab.

The initial dose of study medication will be 600 mg of Eculizumab and will be contained in three (3) 30 ml vials. (Each 30 ml vial contains only 27.5 ml of Eculizumab.) The maintenance dose of study medication will be 900 mg of Eculizumab and will be contained in three (4) 30 ml vials. Patients will be infused with study medication at a concentration of 5 mg/ml, in a volume of 120 mls (600 mg) or 180 mls (900 mg). A one-to-one dilution of the vialed material will be made (one part study medication, one part diluent): e.g. 60 mls study medication to 60 mls diluent. Study medication can be mixed with normal saline, ½ normal saline, or D5W.

The study medication is stable for at least 24 hours once diluted. The medication is stable in the three diluents mentioned above whether it is prepared in standard IV bags or glass evacuated bottles. If the study medication is made up more than several hours in advance of a patient visit, then the diluted material should be stored at 4°C and warmed to room temperature prior to administering the infusion. The solution should be allowed to come to room temperature by taking it

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out of the refrigerator. The material must not be heated in a microwave, nor with any heat source other than ambient air temperature.

The study medication and diluent will be injected into a bag or bottle suitable for infusion. The diluted study medication will then be administered intravenously over 30 minutes. At the site's discretion, the diluted study medication may be placed in a syringe-type pump or infused with an infusion pump. The patients will be monitored for one hour after the end of each infusion.

If an adverse event occurs during the administration of the study medication, the infusion may be slowed or stopped at the discretion of the investigator, dependent upon the nature and severity of the event. If the infusion is slowed, the total infusion time may not exceed two (2) hours.

It is not necessary to protect the infusion bags from light while they are being administered to the patient.

6.4 Duration of Treatment

The duration of treatment with study medication will be 10 weeks.

6.5 Premature Termination of Treatment/Patients From the Trial

If a patient discontinues participation in the trial and has not received any study medication, the patient will be replaced. No further protocol-related procedures need to be performed.

If the patient discontinues participation in the trial after being dosed, regardless if they received any study medication, they will be eligible for the intention-to-treat analysis, and the analysis of non-treatment emergent serious adverse events. Every effort should be made to perform all of the procedures in Section 10.1, under Premature Termination Visit.

Some reasons for the premature discontinuation of a patient from the trial are:

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- Patient wishes to withdraw;
- Patient is unable or unwilling to complete follow-up;
- Patient experiences a serious adverse event.
- Lack of Efficacy: defined as a patient who requires transfusions on two (2) separate occasions after Week 3.

6.6 Drug Accountability

When a drug shipment is received from Clinical Trial Services the investigator or his or her designee will sign the Receipt of Shipment form provided with the shipment and maintain the original copy for collection by the Study Monitor/Research Associate. The duplicate copy should be kept with the pharmacy's dispensing logs.

Each shipment of investigational drug supplies for a study will contain a drug accountability ledger to assist the pharmacist in maintaining current and accurate inventory records covering receipt, dispensing, and the return of study drug supplies. During the course of the study, the following information must be noted in the drug dispensation log: the identification numbers and initials of subjects to whom drug is dispensed, the date(s) that drug was dispensed to subjects, and the date(s) and the number of returned drug vials. Empty vials and vials with residual materials should be kept for inspection by the Study Monitor/Research Associate. The inventory records also must be readily available for inspection by the Study Monitor/Research Associate, and may be subject to FDA or the local regulatory agency inspection at any time.

All used and partially used vials of study medication will be retained until the Study Monitor/Research Associate has reviewed all study drug dispensation logs. The Study Monitor/Research Associate will assure proper documentation exists for disposition of used (empty), partially used and unused vials on-site. The Study Monitor/Research Associate will collect the Drug Dispensing Logs/Inventory Sheets, and copies of these documents will be retained by the investigator for his or her files.

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6.7 Concomitant Therapy

Patients will be treated in accordance with the standard medical practice of the physician in charge of their care, with the following exceptions:

1. Immunosuppressants are allowed, but must be kept stable during the study (Visits 1-12).
2. Iron (Fe) supplements are allowed, but must be kept stable during the study (Visits 1-12).
3. Warfarin is allowed, but may not be started during the study (Visits 1-12).
4. Whole blood or fresh frozen plasma should not be given during the study (Visits 1-12)

All concomitant medications taken by the patient within the thirty days prior to screening will be recorded in the appropriate section of the case report form.

It is recommended that patients are transfused only if their hemoglobin falls to below 10 g/dl and they have symptoms attributable to anemia. Blood for transfusion should not contain complement and hence packed cells in Optimal Additive Solution (OAS) is recommended. Whole blood should not be used.

7. WARNINGS/PRECAUTIONS

To date there have been two cases of urticaria (immediate hypersensitivity reactions) reported during administration of Eculizumab. Both patients experienced a reaction upon receiving their first infusion and both recovered fully after treatment with an antihistamine. Neither patient had hemodynamic instability or breathing difficulties, although one patient had a tingly sensation in the tongue. The patients were discontinued from their respective studies. An acute allergic reaction should be treated according to standard medical practice (e.g., with epinephrine or antihistamine).

Inhibition of the late elements of the complement cascade system may predispose patients to infections with N. meningitidis. There has been one reported case of meningitis with this organism that occurred in a patient with

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membranous nephropathy patient who was enrolled in an open-label extension study. This patient had several risk factors for infection in addition to having been treated with Eculizumab. Following antibiotic treatment, the patient recovered with some sequelae (loss of digits). Therefore, to decrease the risk of such possible infection, all patients in this study must be vaccinated against N. meningitidis. These patients must be vaccinated at least two weeks before his or her first dose of study medication. Additionally, all patients will have frequent throat cultures to detect colonization with N. menigitidis.

In addition to the risks with N. meningitidis discussed above, patients may be at an increased risk for other types of infections. Patients must not be dosed if they have a temperature of 38°C (100.4°F) or greater.

As this is a clinical trial, careful observation of the patient is necessary. Any adverse event experienced during the study period must be described in detail, fully evaluated by the investigator, and reported on the appropriate CRF pages regardless of the investigator's perception of the event's relationship to the study material.

8. PRETREATMENT PROCEDURES

8.1 Informed Consent

Prior to the initiation of any invasive testing or protocol mandated procedure, the study will be explained to the patient. If the patient agrees to all of the requirements of the study, and to the Informed Consent, the patient will sign the Informed Consent Form. This shall be done in accordance with local IRB regulations, as well as applicable State/Provincial and Federal Laws (see Appendix A, Section 1).

8.2 Observations and Measurements

Visit 1 (Day -21 to -14 – Screening)

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This is the initial screening visit, which will occur between 14 and 21 days prior to the first administration of study medication. The screening period may be extended to 4 weeks with permission of the sponsor. The following information will be obtained, or procedures performed, at this visit:

1. Signed informed consent will be obtained from each patient (see section 8.1, above).
2. Demographic data and medical/surgical history, including laboratory data for the previous 3 months, hemoglobin levels over the previous 6 months, and the number of transfusions and total number of units of blood transfused over the previous 12 months.
3. Weight in kilograms, sitting heart rate and blood pressure.
4. Complete physical examination.
5. 12-lead electrocardiogram (ECG).
6. Hematology, to include CBC (hemoglobin, hematocrit, RBC, WBC) with differential (segmented neutrophils, bands, eosinophils, basophils, lymphocytes, monocytes) and platelet count.
7. Blood Chemistries, to include blood glucose, BUN, uric acid, total bilirubin (conjugated and uncongugated), alkaline phosphatase, LDH, ALT (SGPT), AST (SGOT), creatinine, inorganic phosphorus, sodium, potassium, chloride, bicarbonate, calcium, albumin, and total protein.
8. Ferritin, vitamin B_{12}, and folate levels.
9. Urinalysis, to include pH, specific gravity, qualitative protein, glucose, ketones, bilirubin, urobilinogen, and microscopic examination of the sediment.
10. Flow cytometry (10 ml EDTA blood), to include RBC CD55 and CD59; PMN CD16, CD66, and CD55; monocyte CD14, CD64, and CD45; and platelet CD61 and CD55.
11. Throat culture for Neisseria.
12. Use of medications presently, and within the past thirty (30) days. This includes over the counter remedies and vitamin/herbal preparations.
13. Hepatitis B and C screen.
14. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).
15. Vaccination against N. meningitidis (at least two weeks prior to first dose of study medication).

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Visit 2 (Day 0)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following procedures will be completed prior to initial dosing of study medication.

1. Record adverse events that have occurred since Screening.
2. Update concomitant medication record.
3. Sitting heart rate and blood pressure.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9 above).
8. Reticulocyte count.
9. Serum haptoglobin.
10. Plasma (free) hemoglobin.
11. Ham Test.
12. Flow cytometry (see section 8.2, point 10 above).
13. EORTC QLQ-C30.
14. Self assessment of urine diary.
15. Throat culture for Neisseria.
16. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters will be collected (see Appendix B).
17. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
18. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

9. DURING TREATMENT PROCEDURES

9.1 Observations and Measurements

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All study visits and study-associated procedures must occur within +/- 2 days of Visits 3 thru 13.

Visit 3 (Day 7)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visit 4 (Day 14)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Serum haptoglobin.
9. Flow cytometry (see section 8.2, point 10 above).
10. Self assessment of urine diary.
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should

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remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 5 (Day 21)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Coagulation profile, to include PT, PTT, INR.
7. Reticulocyte count.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters will be collected (see Appendix B).
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 6 (Day 28)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9, above).
8. Reticulocyte count.

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9. Serum haptoglobin.
10. Flow cytometry (see section 8.2, point 10 above).
11. EORTC QLQ-C30.
12. Self assessment of urine diary.
13. Throat culture for Neisseria.
14. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
15. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
16. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 7 (Day 35)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7 above).
6. Reticulocyte count.
7. Flow cytometry (see section 8.2, point 10 above).
8. Interval samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
9. Self assessment of urine diary.

Visits 8 (Day 42)

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Reticulocyte count.

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7. Serum haptoglobin.
8. Flow cytometry (see section 8.2, point 10 above).
9. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
10. Self assessment of urine diary.
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 9 (Day 56)

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Coagulation profile, to include PT, PTT, INR.
7. Urinalysis (see section 8.2, point 9, above).
8. Reticulocyte count.
9. Serum haptoglobin.
10. Flow cytometry (see section 8.2, point 10 above).
11. EORTC QLQ-C30.
12. Self assessment of urine diary.
13. Throat culture for Neisseria.
14. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
15. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Visits 10 (Day 70)

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The following observations/procedures will be performed:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.
4. Hematology (see section 8.2, point 6 above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Reticulocyte count.
7. Serum haptoglobin.
8. Flow cytometry (see section 8.2, point 10 above).
9. Self assessment of urine diary.
10. Trough and peak samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
11. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

10. POST-TREATMENT PROCEDURES

10.1 Observations and Measurements

All study visits and study-associated procedures must occur within +/- 2 days of the scheduled visit day for Visit 14.
Patients who drop from the study before Visit 14 should perform all evaluations listed under PT.

Visit 11 (Day 84)
The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Complete physical examination.
2. Sitting heart rate and blood pressure.
3. 12-lead electrocardiogram (ECG).

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4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Ferritin, vitamin B_{12}, and folate levels.
7. Coagulation profile, to include PT, PTT, INR.
8. Urinalysis (see section 8.2, point 9, above).
9. Reticulocyte count.
10. Serum haptoglobin.
11. Plasma (free) hemoglobin.
12. Ham Test.
13. Flow cytometry (see section 8.2, point 10 above).
14. EORTC QLQ-C30.
15. Self assessment of urine diary.
16. Throat Culture for Neisseria.
17. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (see Appendix B).
18. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
19. The occurrence of adverse events will be noted.
20. Record the use/administration of concomitant medications.
21. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).

Patients who **are** participating in the extension study will also have the following observations/procedures performed:

1. Signed informed consent will be obtained from each patient (see section 8.1, above).
2. Weight in kilograms.
3. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Patients who **are not** participating in the extension study will also have the following observations/procedures performed:

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1. Exchange transfusion **if** the PNH RBC's are >50% of the total RBC's by flow cytometry.

Visit 12 (Day 112)

The following observations/procedures will be performed for **all patients** whether or not participating in the extension study:

1. Sitting heart rate and blood pressure.
2. The occurrence of adverse events will be noted.
3. Record the use/administration of concomitant medications.

The following observations/procedures will be performed only on patients **participating** in the extension study:

1. Hematology (see section 8.2, point 6, above).
2. Blood Chemistries (see section 8.2, point 7, above).
3. Urinalysis (see section 8.2, point 9, above).
4. Dosing – Once all of the above procedures/observations have been performed, the study medication may be administered. Patients should remain at the clinic for one hour following the end of the 30 minute infusion for observation.

Premature Termination (PT) Visit

The EORTC QLQ-C30 must be administered **before** any study procedures or any evaluations by the study medical personnel.

The following observations/procedures will be performed:

1. Complete physical examination.
2. Sitting heart rate and blood pressure.
3. 12-lead electrocardiogram (ECG).
4. Hematology (see section 8.2, point 6, above).
5. Blood Chemistries (see section 8.2, point 7, above).
6. Ferritin, vitamin B_{12}, and folate levels.
7. Coagulation profile, to include PT, PTT, INR.

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8. Urinalysis (see section 8.2, point 9, above).
9. Reticulocyte count.
10. Serum haptoglobin.
11. Plasma (free) hemoglobin.
12. Ham Test.
13. Flow cytometry (see section 8.2, point 10 above).
14. EORTC QLQ-C30.
15. Self assessment of urine diary.
16. Throat Culture for Neisseria.
17. Trough samples for the determination of pharmacokinetic, pharmacodynamic, and C5b-9 parameters (See Appendix B).
18. Serum for HAHA (anti-h5G1.1-mAb) (see Appendix B).
19. The occurrence of adverse events will be noted.
20. Record the use/administration of concomitant medications.
21. All women aged 55 years or less will have a serum β-HCG pregnancy evaluation to be performed at the local laboratory, unless surgically sterile (documented).
22. Exchange transfusion **if** the PNH RBC's are >50% of the total RBC's by flow cytometry.

11. ADVERSE EVENTS

Information regarding adverse events (AEs) will be collected from the time the patient signs the informed consent form up to and including the visit 12.

11.1 Safety Definitions

Adverse Event: This is defined as any undesirable clinical experience occurring to a patient whether or not it is considered related to the investigational product.

Further, this undesirable experience should not be the condition that qualified the patient for participation in the study, i.e. PNH. A patient who experiences an exacerbation of PNH that is severe enough to warrant dropping from the study should be withdrawn from the study for lack of efficacy. Additionally, should the exacerbation be so severe as to require the patient's hospitalization, or

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prolongation of hospitalization, the event must be reported as a serious adverse event (SAE). See below for description of an SAE.

Undesirable experiences do however, include exacerbations of other pre-existing conditions, (i.e. other than PNH). That is, if a condition that was present prior to the start of the study occurs more frequently or with greater severity during the study, it constitutes an AE.

Detection

Adverse events may be volunteered spontaneously by the subject, be discovered as a result of general questioning by the Investigator, by physical examination, by ECG, and by laboratory. During all occasions of contact with the Investigator, subjects will be systematically observed and questioned regarding the occurrence of adverse events. Questions will be phrased so that they do not, by suggestion, "lead" the subject into giving information that is not valid.

Non-Treatment Emergent Adverse Events

When an AE occurs after written informed consent has been obtained, but prior to the first dose of study drug, or when the AE occurs more than 30 days after the last dose of study medication the AE will be considered a **non-treatment-emergent AE**. Only serious, non-treatment-emergent AEs that are related to study procedures will be collected.

If a patient dies within 30 days of the last dose of study medication however, the death will be reported as a SAE, regardless of causality.

Treatment Emergent Adverse Events

When an AE occurs between the time the patient receives his or her first dose of study medication and a) his or her exit from the study or b) 30 days after the last dose of study medication, the AE will be considered a **treatment-emergent AE**, except as noted above.

Immediately Reportable Adverse Event (IRAE): This is an AE that must be reported to Alexion Pharmaceuticals within 24 hours of the study center being

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informed of the AE. Immediately reportable AEs include all serious AEs, as listed below, **and** AEs that result in a patient's withdrawal from the study.

Serious Adverse Event: As provided by the ICH criteria, any AE that:

- results in death;
- is life threatening;

(Note: The term "life threatening" refers to an AE that, as it occurs, puts the patient at immediate risk of death. It does not refer to an AE that hypothetically might have caused death if it were more severe.)

- results in hospitalization or prolongation of current hospitalization (not including hospitalization for a pre-existing condition that has not increased in severity or frequency from the patient's underlying medical condition prior to entry into the study);
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/ birth defect in the offspring of a patient; or
- is judged to be medically significant.

(Note: A medically significant AE is a medical event that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or require intervention to prevent one of the outcomes listed above. Medical and scientific judgment should be exercised in deciding whether other AEs appropriately meet these criteria and are immediately reportable to the Sponsor or designee.)

When completing the appropriate forms for reporting the AE, the investigator will be asked to assess the AE as follows:

Severity of Adverse Event: Refers to the extent to which an AE affects the patient's daily activities. Severity will be categorized according to the following criteria:

Mild:	Normal activities unaltered or annoying, but tolerable.
Moderate:	Normal activities altered or require intervention.
Severe:	Unable to undertake normal activities or incapacitated.

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Causality of Adverse Events: Refers to the relationship of the AE to study drug.

Causality will be assessed by the investigator and categorized according to the following criteria:

Unlikely: There is no medical evidence to suggest that the AE is related to study medication usage, or there is a more probable medical explanation.

Possible: There is medical evidence to suggest that the AE is related to study drug usage. However, other medical explanations cannot be excluded as a possible cause.

Probable: There is strong medical evidence to suggest that the AE is related to study drug usage.

11.2 Reporting Adverse Events

When an AE is suspected, all relevant evaluations will be performed and appropriate treatment provided. For non-serious AEs that continue past the last study visit, the subject will be contacted by telephone seven days following their last visit to determine if the event has resolved, is ongoing or has worsened. If the severity and frequency of the AE(s) has not changed, it will be marked as 'Ongoing" on the CRF. If the event has worsened the subject will remain under medical supervision until the investigator and the Sponsor's medical monitor and/or clinical safety director deem the AE to be resolved, stabilized, or no longer serious enough to warrant follow-up. For SAEs that continue past the last study visit, the subject will be contacted by telephone thirty days following their last visit to determine if the event has resolved, is ongoing or has worsened. If the event has worsened the subject will remain under medical supervision until the investigator and the Sponsor's medical monitor and/or clinical safety director deem the SAE to be resolved, stabilized, or no longer serious enough to warrant follow-up.

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All AEs, both serious and non-serious will be recorded on the Adverse Event Log of the CRF.

All Adverse Events

Serious non-treatment-emergent AEs related to study procedures and all treatment-emergent AEs (serious and non-serious) will be reported on the appropriate tabs on the CRF.

Serious Adverse Events

Information regarding serious AEs will be recorded on the appropriate tabs of the CRF.

In addition, the investigator or designee must complete the IRAE section of the CRF, as well as the Concomitant Medications and Demographics sections. These forms must be completed and Alexion Pharmaceuticals notified within 24 hours of the study center being informed of the SAE.

Withdrawals Due to Adverse Events

When study medication is discontinued due to an AE, or the patient withdraws from the study due to an AE, whether or not the event was related to study medication, the investigator or designee must complete the Adverse Event Log CRF, and the Patient Accountability, Concomitant Medications and Demographics sections of the CRF. The investigator must Alexion Pharmaceuticals within 24 hours of the study center being informed of the AE.

11.3 Exposure in Utero

If it becomes known during the administration of study medication that a patient is pregnant, the study medication will be stopped. In addition, the study medication will be discontinued for any woman who becomes pregnant during the extension study. Alexion Pharmaceuticals should be notified, and will supply the investigator with a copy of an "Exposure in utero" form. The patient should be followed until the outcome of the pregnancy is known, at which time the form should be completed

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and returned to the Sponsor. If additional follow-up is required, the investigator will be informed.

11.4 Adverse Event Reporting Contacts

For Immediately Reportable Adverse Events (IRAEs), the investigator should contact Alexion Pharmaceuticals at the following numbers:

North America
Telephone Number: 011-203-271-8215 or 011-203-272-2596
Fax Number: 011-203-271-8196

12. CRITERIA FOR THE EVALUATION OF STUDY RESULTS

12. 1 Criteria for the Evaluation of Efficacy

- As this is a pilot study, there is no formal primary efficacy endpoint.

The secondary endpoints comprise changes in:

- Number of units of PRBCs transfused.
- Time to first transfusion of PRBCs.
- Change from baseline hemoglobin and hematocrit.
- Change from baseline of indicators of hemolysis including reticulocyte count, haptoglobins, LDH and bilirubin.
- Number of paroxysms and time to first paroxysm (defined as urine of color grade $\geq$ 6; See Appendix D).
- Reticulocyte count.
- Serum haptoglobin
- Change from baseline EORTC QLQ-C30
- The extent and duration of complement inhibition (pharmacodynamics).

The exploratory endpoints consist of changes in:

- Platelet and neutrophil count

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- Change in the size of the GPI-deficient (PNH) clone, assessed by flow cytometry (including RBC's, PMN's, monocytes and platelets).
- Ham test
- Plasma (free) hemoglobin

12.2 Criteria for the Evaluation of Safety

Safety will be measured by collecting data on adverse events and laboratory values. A complete laboratory evaluation (hematology, blood chemistries and urinalysis) will be done at screening, pre-dose, during the active treatment period (days 0-84), and at study termination (day 84). Specific attention will be given to the occurrence of infection.

12.3 Evaluation of Pharmacokinetic and Pharmacodynamic Data

The trough concentrations of Eculizumab collected from patients at Visits 2 through 12 will be used to calculate the pharmacokinetic profile of the drug in patients with PNH. Similarly, the pharmacodynamics of Eculizumab as regards the degree and duration of the blockade of C5 will also be determined. Data will be compared to a preliminary pharmacokinetic/pharmacodynamic model of Eculizumab, which has been generated from other Phase I data. The analysis has been, and will be, performed with the USC-PACK suite of PK analysis tools (using both the iterative two- stage parametric Bayesian (IT2B) as well as the Non-parametric Expectation Maximization (NPEM) methods).

12.4 Evaluation in Case of Discontinued Treatment

As all patients who receive any amount of study medication are eligible for safety and intention to treat analyses, all observations and measurements, as outlined in Sections 8, 9, and 10, above, should be performed. If a patient drops out of the study all procedures listed under PT Visit should be performed at the termination visit.

12.5 Removal of Patients from Study or Analysis

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Patients may be removed from the study because of serious adverse events, as well as conditions or intercurrent illnesses that preclude compliance with the protocol from the standpoint of a patient's safety or well-being. Patients may also be removed because of translocation to a different geographical area or otherwise being lost to follow-up.

If a patient is withdrawn from the study after receiving any amount of study medication, he or she will be considered as having prematurely terminated the study and will not be replaced. All such patients will be eligible for the safety and intention-to-treat analyses.

12.6. Evaluation of Data for the Safety and Protection of Subjects

Due to the small size and short duration of this study, no data and safety monitoring board will be used.

12.7 Stopping Rules

None

13. DATA PROCESSING AND ANALYSIS

13.1 Sample Size Estimation

Not applicable, as this is a pilot study.

13.2 Statistical and Analytical Plan

Demographics and Baseline Data

Demographics data and other baseline data will be examined and presented as a single group. Summary statistics such as means, medians, and standard deviations for continuous data, and percentages for categorical data will be used to present distributions of patient characteristics.

Efficacy Assessments

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Due to the small sample size, no formal statistical analyses will be performed.

Summary statistics (frequency and percentage for categorical endpoint, mean, median, standard deviation, and range for continuous variable) will be presented for pretreatment vs. post-treatment values for all secondary and exploratory endpoints. The difference in the incidence of outcome among treatment groups will be tested using Fisher's Exact test, or Chi-square statistics. The difference in the mean, median or scores of the outcome measures among the treatment groups will be tested using T-test, Analysis of Variance (or Wilcoxon rank sum test and Kruskal-Wallis test for data with non-normal distribution).

Safety

Any patient who receives any amount of study medication and has post baseline safety data is included for safety evaluation.

Safety will be assessed by examination of adverse events, clinical laboratory data, and vital signs. Safety parameters will be assessed during the study period including the follow-up period.

Adverse events (both patient and event counts) will be tabulated by severity and, for each severity, by investigator-assessed relationship to study drug. In addition, adverse events related to the occurrence of infection will be tabulated separately.

Hematology, blood chemistry, and urinalysis data will be analyzed using distribution shift tables. Laboratory values will be categorized as normal, abnormally low, or abnormally high at baseline and at post-baseline assessments. In addition, mean changes from pre-infusion to post-dose assessments will be summarized by treatment group using the mean, median, standard deviation, and range.

Data from the pharmacodynamic and complement assays (PD, C5b-9) will be presented using summary statistics such as mean, median, standard deviation and range at each post-baseline assessment. Vital signs (blood pressure, heart

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rate, and temperature) will be summarized by the mean change from baseline to post-baseline assessments. In addition the median, standard deviation, and range at each post-baseline assessment will be presented.

Pharmacokinetic Parameters

Since only trough samples will be used, maximum plasma concentration (Cmax), time to maximum concentration (Tmax), terminal rate constant and half-life, area under the serum concentration versus time curve (AUC), and apparent column distribution and apparent clearance will not be calculated.

13.3 Interim Analysis

An interim analysis is not foreseen.

13.4 Use of Data and Publication

Please refer to the terms outlined in your study specific Clinical Study Agreement or Master Clinical Study Agreement.

14. ESTIMATED DURATION OF THE STUDY

The estimated duration of the study, given a three month enrollment period, is 7 months.

15. PREMATURE TERMINATION OF THE STUDY

Please refer to the terms outlined in your study specific Clinical Study Agreement or Master Clinical Study Agreement.

16. PHARMACOKINETICS AND PHARMACODYNAMICS

Assays for anti-human antibodies (HAHA) and C5b-9 will be performed at the laboratories of Alexion Pharmaceuticals. Pharmacokinetic and pharmacodynamic assays will also be performed at Alexion Pharmaceuticals.

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Samples at the times specified for assays in Figure 1 should be taken as detailed in Appendix B, and labeled with the labels provided.

Shipment of Frozen Samples

All frozen samples for h5G1.1-mAb neutralizing factor (HAHA), C5b-9, pharmacodynamics and pharmacokinetics will be shipped to

Michael Straub
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410.

Please refer to the shipping instructions as detailed in Appendix C, and in the Clinical Laboratories Manual and Clinical laboratories summary chart.

17. PROJECT MONITORS

The Clinical Project Director for this study will be:

Christopher F. Mojcik M.D., Ph.D.
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
(203) 272-2596 (office)
(203) 784-4333 (pager)

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18. STUDY TIMETABLE

Projected starting date: 05/01/02
Projected number of subjects entered: 10
Projected number of subjects valid for efficacy analyses: 10
Projected completion date: 11/31/02

The following documents must be submitted to Alexion Pharmaceuticals, Inc. prior to the start of the study:

- Curriculum Vitae (Principal and Sub-Investigators)
- Institutional Review Board (IRB) Approval
- Copy of the IRB Approved Informed Consent Form
- Signed copy of protocol
- Signed copy of the FDA 1572 Form (USA IND Studies)
- Qualified Investigator Undertaking (Canadian sites only)
- Research Ethics Board Attestation (Canadian sites only)

THIS CONFIDENTIAL INFORMATION ABOUT AN INVESTIGATIONAL DRUG IS PROVIDED FOR THE EXCLUSIVE USE OF INVESTIGATORS OF THIS DRUG AND IS SUBJECT TO RECALL AT ANY TIME. THE INFORMATION IN THIS DOCUMENT MAY NOT BE DISCLOSED UNLESS SUCH DISCLOSURE IS REQUIRED BY FEDERAL OR STATE LAW OR REGULATIONS SUBJECT TO THE FOREGOING. THIS INFORMATION MAY BE DISCLOSED ONLY TO THOSE PERSONS INVOLVED IN THE STUDY WHO HAVE A NEED TO KNOW WITH THE OBLIGATION NOT TO FURTHER DISSEMINATE THIS INFORMATION. THESE RESTRICTIONS ON DISCLOSURE WILL APPLY EQUALLY TO ALL FUTURE ORAL OR WRITTEN INFORMATION SUPPLIED TO YOU BY ALEXION, WHICH IS DESIGNATED AS "PRIVILEGED" OR "CONFIDENTIAL".

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19. REFERENCES

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APPENDIX A

Ethical and Regulatory Considerations

The following must be observed to comply with regulatory agencies' regulations for the conduct and monitoring of clinical investigations; they also represent sound clinical research practice:

A. Informed Consent

The principles of informed consent are described in ICH Guideline "Good Clinical Practice: Consolidated Guideline", and other federal regulatory agency documents as listed in section 4. They must be followed to comply with Food and Drug Administration and other local regulatory agencies' regulations for the conduct and monitoring of clinical investigations. In seeking informed consent, the following information shall be provided in a language understandable to the subject.

1. The following are the basic elements of informed consent, which should be provided to each subject:

   a. A statement that the study involves research, an explanation of the purposes of the research and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental.
   b. A description of any reasonably foreseeable risks or discomforts to the subject.
   c. A description of any benefits to the subject or to others which may reasonably be expected from the research.
   d. A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject.
   e. A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained and that notes the possibility that the regulatory agencymay inspect the records.
   f. For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained.
   g. An explanation of whom to contact for answers to pertinent questions about the research and research subjects' right, and whom to contact in the event of a research-related injury to the subject.
   i. A statement that participation is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

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2. When appropriate, one or more of the following elements of information shall also be provided to each subject:

   a. A statement that the particular treatment or procedure may involve risks to the subject (or to the embryo or fetus, if the subject is or may become pregnant) which are currently unforeseeable.
   b. Anticipated circumstances under which the subject's participation may be terminated by the investigator without regard to the subject's consent.
   c. Any additional costs to the subject that may result from participation in the research.
   d. The consequences of a subject's decision to withdraw from the research and procedures for orderly termination of participation by the subject.
   e. A statement that significant new findings developed during the course of the research which may relate to the subject's willingness to continue participation will be provided to the subject.
   f. The approximate number of subjects involved in the study.

A subject (or the subject's legally authorized representative) must give his/her written consent to participate in the study. This consent must be witnessed, dated and retained by the investigator as part of the study records. A copy of the consent form must be given to the subject.

If Experimental Subject's Bill of Rights is applicable (as of January 1988, only applicable in the State of California), this form must also be prepared and signed by each subject and retained as part of the required study records. A copy of the Bill of Rights must be given to the subject.

A copy of the proposed consent form must be submitted to the Institutional Review Board together with the protocol for approval. A copy of the IRB approved consent form must be available for inspection by the Alexion Study Monitor/Research Associate (or monitors/associates contracted by Alexion), and evidence of an IRB approved consent form may be required prior to shipment of drug supplies to the investigator. Each subject's signed informed consent form must be kept on file by the investigator for regulatory agency/Monitor inspection at any time.

B. Institutional Review

The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board, as defined by ICH Guideline "Good Clinical Practice: Consolidated Guideline", and other federal regulatory agency documents as listed in section 4)
A copy of the letter of approval from the Board (Research Ethics Board Attestation in Canada), which should contain specific identification of the documents approved, must be received by the Study Monitor/Research Associate at Alexion Pharmaceuticals or its designee prior to shipment of drug supplies to the investigator.

All changes to approved protocols, including amendments and change of principal investigator, must be done in writing and be approved by the Board prior to implementation, and documentation of this approval provided to the Study Monitor/Research Associate. If there is any amendment to protect subjects from an

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immediate hazard, it may be implemented immediately, and must be reported to the IRB in writing within 10 working days. The IRB still must approve the amendment. Records of the Institutional Review Board review and approval of all documents pertaining to this study must be kept on file by the investigator and are subject to Alexion or regulatory agency inspection at any time during the study.

Periodic status reports must be submitted to the IRB, at least yearly, as well as notification of completion of the study. A copy of these reports should be sent to the Study Monitor/Research Associate. The investigator must maintain an accurate and complete record of all reports and documents submitted to the IRB.

C. Recording of Data

Data for individual subjects are to be recorded on remote data entry screens, as designed by Alexion Pharmaceuticals.

A set of RDE screens must be completed for each subject enrolled, including those removed from the study. If a subject is removed from study, the reason for removal must be noted on the appropriate RDE screen by the investigator or his/her designee. The principal investigator must review all screens and will indicate, by his or her signature at the end of the trial, the veracity of the information contained therein.

RDE screens must be kept current to reflect subject status at each phase during the course of the study. Subjects are not to be identified on the screens by name; appropriate coded identification and patient initials must be used. The investigator/designee must keep a separate log of patient names and addresses. If requested during a regulatory agency inspection, this log may be shown to the regulatory agency investigator but no copy should be provided.

The RDE data, appropriate source documentation and copies of test results must be available at all times for inspection by the Study Monitor/Research Associates and regulatory agency.

D General Information

1. Study records including case report forms, signed FDA 1572, Qualified Investigator Undertaking in Canada, originals of test results, signed informed consent forms, Institutional Review Committee approval letters (Research Ethics Board Attestation in Canada), copies of documents submitted to the IRB, and other documents pertaining to the conduct of the study must be kept on file by the investigator. If the investigator wishes to assign the files to someone else or remove them to another location he should inform the Alexion Pharmaceuticals monitor in writing about this change. Study records are subject to FDA inspection at any time. Study records must be maintained for a minimum of two years following written notification by Alexion Pharmaceuticals of either regulatory agency marketing authorization or discontinuance of the IND.

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2. The Study Monitor/Research Associate must be acquainted with the investigator and other key people involved in the study. Monitoring visits will be scheduled periodically and regularly. It is essential that the investigator and the designated study coordinator set aside a sufficient amount of time for these visits to permit an adequate review of the study's progress and of completed case reports.

3. If there is a change or addition of sub-investigator, the FDA 1572 form and Qualified Investigatory Undertaking in Canada must be promptly amended.

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APPENDIX B

PROCEDURE FOR COLLECTING STUDY C02-001 CLINICAL RESEARCH SAMPLES

PD – Pharmacodynamic – Hemolysis, PK – Pharmacokinetic, and HAHA – Human Anti-Human Antibody Assays

1. **Serum** is used in these assays.
2. Blood is drawn into tubes that **do not** contain anticoagulant.
3. Blood is allowed to clot at room temperature for 30 minutes to 1 hour.
4. Centrifuge the tubes.
5. Remove serum and place at least 1ml into labeled tube(s). **Please note, for visit 2, 5 tubes with at least 1 ml of serum per tube is required for PK.**
6. The serum containing tubes are stored at –70°C until they are shipped to Alexion Pharmaceuticals, Inc.

PD – Pharmacodynamic – C5b-9 Complement Assay

1. **Plasma** is used in this assay.
2. Blood is drawn into EDTA-containing tubes.
3. Centrifuge the tubes immediately (within 2 hours).
4. Remove the plasma and place at least 1 ml into a labeled vial.
5. The serum containing tubes are stored at –70°C until they are shipped to Alexion Pharmaceuticals, Inc.

Note: The Serum and Plasma may be maintained up to 4 hours at 2-8°C (refrigerated or on wet ice) prior to final storage at –70°C.

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APPENDIX C
Study C02-001 Clinical Research Sample Shipping Instructions

PK	Pharmacokinetics
PD	Pharmacodynamics – Hemolysis
C5b-9	Pharmacodynamics – C5b-9 Complement Assay
HAHA	Human Anti-Human Antibody – Neutralizing Antibody

Samples Labeled PK, PD, HAHA, and C5b-9

1. Ship by patient, once the patient has completed the study, to Alexion Pharmaceuticals, Inc.
2. Pack all samples frozen on dry ice

Michael Straub

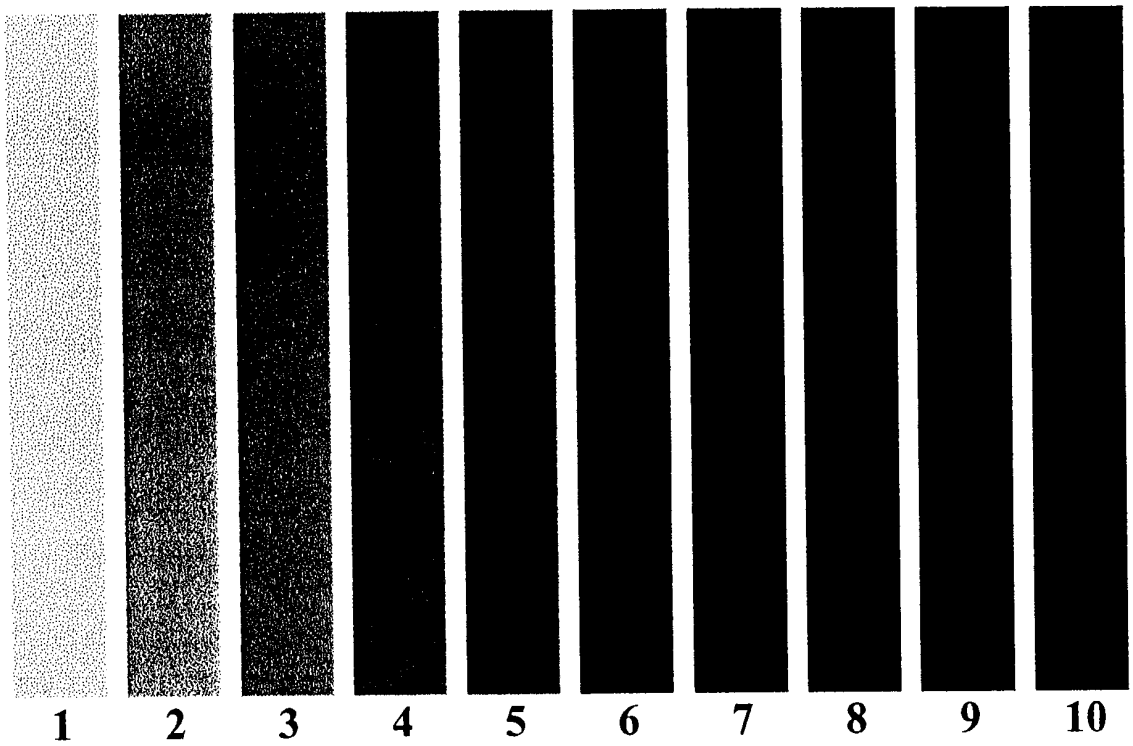
Alexion Pharmaceuticals, Inc.

352 Knotter Drive
Cheshire, CT 06410

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APPENDIX D

Paroxysm Grading



Colour of urine first time in the morning

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CONFIDENTIAL DISCLOSURE AGREEMENT

CONFIDENTIAL DISCLOSURE AGREEMENT (the "Agreement"), dated as of January 11, 2002, between Alexion Pharmaceuticals, Inc., with offices at 352 Knotter Drive, Cheshire Connecticut 06410 ("Alexion"), and Dr. Peter Hillmen, with offices at Haematological Malignancy Diagnostic Service, Algernon Firth Building, Leeds General Infirmary, Great George Street, Leeds, UK LS1 3EX (the "Receiving Party").

WHEREAS, Alexion possesses certain valuable and confidential information relating to the research and development, clinical development, therapeutic opportunities (including PNH) and business matters involving its proprietary antibody candidate referred to as h5G1.1-mAb (the "Information"); and

WHEREAS, Alexion and the Receiving Party wish to explore the possibility of entering into certain arrangements relating to the Information;

NOW, THEREFORE, in consideration of the mutual promises contained herein, Alexion and the Receiving Party hereby agree as follows:

1. Alexion shall disclose such of the Information as it deems necessary and the Receiving Party shall accept and hold the Information in confidence in accordance with the provisions of Paragraph 2 hereof.

2. The Receiving Party agrees not to (1) disclose to any third party any or all of the Information, or permit any third party to have access to such Information, or (2) use such Information for any purpose other than to evaluate the Information in connection with discussions between the parties. In addition, the Receiving Party agrees to disclose the Information only to those of its employees who shall reasonably need to know the Information in order to evaluate the Information and who are bound to terms of confidentiality that are substantially as protective as the terms hereof. However, such obligations assumed by the Receiving Party shall not apply to any of the following:

a. Information which is in the public domain at the time of disclosure hereunder or which subsequently comes within the public domain through no fault of or action by the Receiving Party; and

b. Information which is in the possession of the Receiving Party at the time of disclosure by Alexion or which is independently discovered, after the date hereof, by the Receiving Party without the aid, application or use of the Information, in each such case as evidenced by written records; and

c. Information which is obtained, after the date hereof, by the Receiving Party from any third party that is lawfully in possession of such Information and not in violation of any contractual or legal obligation with respect to such Information.

3. This Agreement, including the confidentiality and limited use obligations hereunder, shall remain in effect for a period of seven (7) years from the date of disclosure of the Information.

4. The Receiving Party shall return to Alexion, upon request, any and all written documents and samples of any materials provided by Alexion hereunder, except that the Receiving Party may retain one copy for its Legal Division for record keeping purposes only.

5. No right or license, either express or implied, with respect to the Information is granted hereunder by Alexion to the Receiving Party. The disclosure of the Information by Alexion to the Receiving Party shall not result in any obligation on the part of either party to enter into any future agreement relating to the Information or to undertake any other obligation not set forth in a written agreement signed by the parties hereto.

6. This Agreement contains all the representations and agreements between the parties relating to the Information and any representation, promise or condition concerning the same which is not contained herein or in a superseding written agreement referring to this Agreement shall not be binding on either party hereto.

7. This Agreement shall be governed by and construed in accordance with the laws of the State of Connecticut (as the same would be applied to agreements entered into in Connecticut by two residents thereof), and each party agrees to the jurisdiction of the State and federal courts of Connecticut.

IN WITNESS WHEREOF, the Receiving Party and Alexion have caused this Agreement to be executed by their respective duly authorized representatives as of the date first above written.

ALEXION PHARMACEUTICALS, INC.

By: _____
Name: T. Dubin
Title: VP + General Counsel

_____
Dr. Peter Hillmen

CDA _ Dr. Hillmen 1-10-02

Form of Indemnity for Clinical Studies

To: The Leeds Teaching Hospitals NHS Trust, Beckett Street, LEEDS LS9 7TF ("the Authority")

From: Alexion Pharmaceuticals, Inc. ("the Sponsor")

Re: Clinical Study No C02-001 with Eculizumab

1 It is proposed that the Authority should agree to participate in the above sponsored study ("the Study") involving patients of the Authority ("the Subjects") to be conducted by Dr. Peter Hillmen (the "Investigators") in accordance with the protocol annexed, as amended from time to time with the agreement of the Sponsor and the Investigator ("the Protocol"). The Sponsor confirms that it is a term of its agreement with the Investigators that the Investigators shall obtain all necessary approvals of the applicable Local Research Ethics Committee and shall resolve with the Authority any issues of a revenue nature.

2 The Authority agrees to participate by allowing the Study to be undertaken on its premises utilising such facilities, personnel and equipment as the Investigators may reasonably need for the purpose of the Study.

3 In consideration of such participation by the Authority, and subject to paragraph 4 below, the Sponsor indemnifies and holds harmless the Authority and its employees and agents against all claims and proceedings (to include any settlements or ex-gratia payments made with the consent of the parties hereto and reasonable legal and expert costs and expenses) made or brought (whether successfully or otherwise):

3.1 by or on behalf of Subjects taking part in the Study (or their dependants) against the Authority or any of its employees or agents for personal injury (including death) to Subjects arising out of or relating to the administration of the product(s) under investigation or any clinical intervention or procedure provided for or required by the Protocol to which the Subjects would not have been exposed but for their participation in the Study.

3.2 by the Authority, its employees or agents or by or on behalf of a Subject for declaration concerning the treatment of a Subject who has suffered such personal injury.

4 The above indemnity by the Sponsor shall not apply to any such claim or proceeding:

4.1 to the extent that such personal injury (including death) is caused by the negligent or wrongful acts or omissions or breach of statutory duty of the Authority, employees or agents;

4.2 to the extent that such personal injury (including death) is caused by the failure of the Authority, its employees, or agents to conduct the Studies in accordance with the Protocol;

4.3 unless as soon as reasonably practicable following receipt of notice of such claim or proceeding, the Authority shall have notified the Sponsor in writing of it and

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shall, upon the Sponsor's request, and at the Sponsor's cost, have permitted the Sponsor to have full care and control of the claim or proceeding using legal representation of its own choosing;

4.4 if the Authority, its employees, or agents shall have made any admission in respect of such claim or proceeding or taken any action relating to such claim or proceeding prejudicial to the defence of it without the written consent of the Sponsor such consent not to be unreasonably withheld provided that this condition shall not be treated as breached by any statement properly made by the Authority, its employees or agents in connection with the operation of the Authority's internal complaint procedures, accident reporting procedures or disciplinary procedures or where such statement is required by law.

5 The Sponsor shall keep the Authority and its legal advisers fully informed of the progress of any such claim or proceeding, will consult fully with the Authority on the nature of any defence to be advanced and will not settle any such claim or proceeding without the written approval of the Authority (such approval not to be unreasonably withheld).

6 Without prejudice to the provisions of paragraph 4.3 above, the Authority will use its reasonable endeavours to inform the Sponsor promptly of any circumstances reasonably thought likely to give rise to any such claim or proceeding of which it is directly aware and shall keep the Sponsor reasonably informed of developments in relation to any such claim or proceeding even where the Authority decides not to make a claim under his indemnity. Likewise, the Sponsor shall use its reasonable endeavours to inform the Authority of any such circumstances and shall keep the Authority reasonably informed of developments in relation to any such claim or proceeding made or brought against the Sponsor alone.

7 The Authority and the Sponsor will each give to the other such help as may reasonably be required for the efficient conduct and prompt handling of any claim or proceeding by or on behalf of Subjects (or their dependants) or concerning such a declaration as is referred to in paragraph 3.2 above.

8 Without prejudice to the foregoing if injury is suffered by a Subject while participating in the Study, the Sponsor agrees to operate in good faith the Guidelines published in 1991 by The Association of the British Pharmaceutical Industry and entitled "Clinical Trial Compensation Guidelines " (where the Subject is a patient) and the Guidelines published in 1988 by the same Association and entitled "Guidelines for Medical Experiments in non patient Human Volunteers" (where the Subject is not a patient) and shall request the Investigator to make clear to the Subjects that the Study is being conducted subject to the applicable Association Guidelines.

9 For the purpose of this indemnity, the expression "agents" shall be deemed to include without limitation any nurse or other health professional providing services to the Authority under a contract for services or otherwise and any person carrying out work for the Authority under such a contract connected with such of the Authority's facilities and equipment as are made available for the Study under paragraph 2 above.

10 This indemnity shall be governed by and construed in accordance with English law.

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SIGNED on behalf of The Leeds Teaching Hospitals NHS Trust

Signature: .....

Date of Signature: 15/07/03 .....

15 July 2003

DR. D. R. NORFOLK
ASSOCIATE R & D DIRECTOR
LEEDS TEACHING HOSPITALS NHS TRUST

Position: **Associate Director of R&D**

SIGNED on behalf of the Company

Signature: .....

Date of signature: 7-3-03 .....

Position: VP TEC .....

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CLINICAL TRIAL SERVICES - DURHAM
ALEXION C02-001

DRUG DESTRUCTION FORM

SITE: 010

PHARMACY CONTACT: Caroline Bedford

PHONE: +44 (0) 113 392 2459

FAX:+44 (0) 113 392 8279

DATE: 3/Apr /2003.

Vial Numbers (Record one Vial # per line)	Empty/Partial/Unused	Date Destroyed	Site Initials/Date
20001	Partial	3/4/03	CB 3/4/03
20002	Empty		
20003	Empty		
20004	Empty		
20005	Empty.		
20006	Partial		
20013	Empty		
20014	Empty.		
20015	Partial		
20029	Empty		

All entries must be made in black ink. Any errors should be crossed out with a single stroke, initialed, and dated. The use of white out, correction tape or scribbling out errors is not permitted. Once complete and verified by the study monitor, please fax a copy of this form to CTS (919) 479-2633.

Method of Destruction: Incineration

PLEASE PLACE COPY OF YOUR STANDARD OPERATING PROCEDURES ON DRUG DESTRUCTION IN THIS BINDER.

Alexion Pharmaceuticals Inc.
352 Knotter Drive ▲ Cheshire, CT ▲ 06410 ▲ Phone 203-272-2596 ▲ Fax 203-271-8196 ▲ www.alxn.com

Alexion Pharmaceuticals, Inc.
EXHIBIT II, ATTACHED TO MASTER CLINICAL STUDY AGREEMENT, FOR SPECIFIC STUDY SCOPE, BUDGET & PAYMENT SCHEDULE WITH ST. GEORGE'S HOSPITAL MEDICAL SCHOOL

Study Specifications

Upon signature, this Exhibit will be incorporated into and made part of the Master Clinical Study Agreement between Alexion Pharmaceuticals, Inc. ("Alexion") and St. George's Hospital Medical School ("Institution") dated May 1, 2002.

1. Product/Compound: h5G1.1-mAb ("Study Drug")

2. Protocol # and Title: C02-001, entitled: "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria" ("Study").

3. Principal Investigator: Judith Marsh, MD

4. Contract Research Organization (if applicable, if not put N/A): Kendle

5. Scope:

   The Study will begin following IRB approval and receipt by Alexion of relevant Study documents. The Study will begin around May 1, 2002 and the anticipated completion date is December 31, 2003. Such dates may be modified by written agreement executed by Principal Investigator and authorized representatives of both Institution and Alexion.

6. Subjects:

   The anticipated number of completed and valid subjects in the Study shall be enrolled within the time frame described above, and according to the criteria listed in the Protocol.





7.2 Definitions:

A "Qualified Study Subject" is a research subject who has been randomized and has met the entrance criteria as defined in the Protocol or who is given written permission by Alexion to enter the Study.

A "Protocol Compliant Study Subject" is a Qualified Study Subject who has met the minimum attendance and compliance standards in the Protocol for evaluation of effectiveness of the Study Drug, and has completed the Study.

A "Non-Protocol Compliant Study Subject" is a Qualified Study Subject that violates the Study procedures, such as, insufficient clinic attendance, poor protocol compliance or other protocol violations, as defined in the Protocol, but the subject completes the Study.

An "Early-Terminated Study Subject" is a Qualified Study Subject who has been withdrawn from the Study because of: lack of efficacy, adverse event, lost to follow-up, or voluntary patient withdrawal. Additional follow-up may be required for these patients to meet FDA regulations.

A "Screen Failure Study Subject" is a study subject who meets all entrance criteria and consents to enroll in the study but fails to randomize due to, e.g., unacceptable laboratory values at the screening visit.

A "Non-Qualified Study Subject" is a research subject who has not met the entrance criteria as defined in the Protocol.







8. Provisions of Master Clinical Study Agreement

The Principal Investigator agrees to be bound by the terms of the Master Clinical Study Agreement between Alexion and Institution dated, May 1, 2002. Without limiting the foregoing, the Principal Investigator agrees to the intellectual property provisions of Section 2 of the Master Clinical Study Agreement and the confidentiality provisions of Section 7 of the Master Clinical Study Agreement.

9. Notices

All communications and notices required or permitted under this Agreement shall be deemed sufficiently served if hand delivered or served by certified mail, postage prepaid, addressed as follows:

TO ALEXION:

Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO ALEXION:
Thomas I. H. Dubin
Vice President and General Counsel
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410

TO PRINCIPAL INVESTIGATOR:
Judith Marsh, MD
St. George's Hospital
Blackshaw Road
London SW17 0QT

TO INSTITUTION:
Mr. Frank Shore, Director of Finance
St. George's Hospital Medical School
Ground Floor Hunter Wing
Cranmer Terrace
Tooting, London SW17 0RE

ACCEPTED BY:

Alexion Pharmaceuticals, Inc.
By: _____
Thomas I. H. Dubin
Vice President and General Counsel

St. George's Hospital Medical School
By: _____
Frank Shore
Director of Finance

The undersigned, as Principal Investigator, hereby agrees to accept responsibility for performance of the Protocol described herein, acknowledges having reviewed this Agreement and agrees to be bound by the terms and conditions stated herein and in the Master Clinical Study Agreement dated, May 1, 2002, which is incorporated herein by reference.

PRINCIPAL INVESTIGATOR

_____
Judith Marsh, MD
5/2/03
Date

FORM OF INDEMNITY FOR CLINICAL STUDIES

To:

Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410 ("the Sponsor")

From: St George's Hospital NHS Trust, Blackshaw Road Tooting, London SW17 0QT and St George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE ("the Authorities")

Re: Clinical Study No: C02-001, "An Open-Label Pilot Study of the Effect of Eculizumab (h5G1.1-mAb) on Patients with Transfusion Dependent, Hemolytic, Paroxymal Nocturnal Hemoglobinuria"

1. It is proposed that the Authorities should agree to participate in the above sponsored study ("the Study") involving patients of the Authorities ("the Subjects") to be conducted by ("the Investigator") in accordance with the protocol annexed, as amended from time to time with the agreement of the Sponsor and the Investigator ("the Protocol"). The Sponsor confirms that it is a term of its agreement with the Investigator that the Investigator shall obtain all the necessary approval of the applicable Local Research Ethics Committee and shall resolve with the Authorities any issues of a revenue nature.

2. The Authorities agrees to participate by allowing the Study to be undertaken on its premises utilising such facilities, personnel and equipment as the Investigator may reasonably need for the purpose of the Study.

3. In consideration of such participation by the Authorities, and subject to paragraph 4 below, the Sponsor indemnifies and holds harmless the Authorities and its employees and agents against all claims and proceedings (to include any settlements or ex-gratis payments made with the consent of the parties hereto and reasonable legal and expert costs and expenses) made or brought (whether successfully or otherwise)

   a) by or on behalf of Subjects taking part in the study (or their dependants) against the Authorities or any of its employees or agents for personal injury (including death) to Subjects arising out of or relating to the administration of the product(s) under investigation or any clinical intervention or procedure provided for or required by the Protocol to which the Subjects would not have been exposed but for their participation in the Study.

   b) by or on behalf of a Subject for a declaration concerning the treatment of a Subject who has suffered such personal injury.

4. The above indemnity by the Sponsor shall not apply to any such claim or proceeding:

   4.1 to the extent that such personal injury (including death) is caused by the negligent or wrongful acts or omissions or breach of statutory duty of the Authorities, its employees or agents.

   4.2 to the extent that such personal injury (including death) is caused by the failure of the Authorities, its employees, or agents to conduct the Study in accordance with the Protocol.

4.3 unless as soon as reasonably practicable following receipt of notice of such claim or proceeding, the Authorities shall have notified the Sponsor in writing of it and shall, upon the Sponsor's request, and at the Sponsor's cost, have permitted the Sponsor to have full care and control of the claim or proceeding using legal representation of its own choosing.

4.4 If the Authorities, its employees, or agents shall have made any admission in respect of such claim or proceeding or taken any action relating to such claim or proceeding prejudicial to the defence of it without the written consent of the Sponsor such consent not to be unreasonably withheld provided that this condition shall not be treated as breached by any statement properly made by the Authorities, its employees or agents in connection with the operation of the Authorities internal complaint procedures, accident reporting procedures or disciplinary procedures or where such statement is required by law.

5. The Sponsor shall keep the Authorities and its legal advisers fully informed of the progress of any such claim or proceeding, will consult fully with the Authorities on the nature of any defence to be advanced and will not settle any such claim or proceeding without written notice to the Authorities.

6. Without prejudice to the provisions of paragraph 4.3 above, the Authorities will use its reasonable endeavours to inform the Sponsor promptly of any circumstances reasonably thought likely to give rise to any such claim or proceeding of which it is directly aware and shall keep the Sponsor reasonably informed of developments in relation to any such claim or proceeding even where the Authorities decides not to make a claim under this indemnity. Likewise, the Sponsor shall use its reasonable endeavours to inform the Authorities of any such circumstances directly involving the Subjects and shall keep the Authorities reasonably informed of developments in relation to any such claim or proceeding made or brought against the Sponsor alone.

7. The Authorities and the Sponsor will each give to the other such help as may reasonably be required for the efficient conduct and prompt handling of any claim or proceeding by or on behalf of Subjects (or their dependants) or concerning such a declaration as is referred to in paragraph 3(b) above.

8. Without prejudice to the foregoing if injury is suffered by a Subject while participating in the Study, the Sponsor agrees to operate in good faith the Guidelines published in 1991 by The Association of the British Pharmaceutical Industry and entitled "Clinical Trial Compensation Guidelines" (where the Subject is a patient) and the Guidelines published in 1988 by the same Association and entitled "Guidelines for Medical Experiments in non-patient Human Volunteers" (where the Subject is not a patient) and shall request the Investigator to make clear to the Subjects that the Study is being conducted subject to the applicable Association Guidelines.

9. For the purpose of this indemnity, the expression "agents" shall be deemed to include without limitation any nurse or other health professional providing services to the Authorities under a contract for services or otherwise and any person carrying out work for the Authorities under such a contract connected with such of the Authorities facilities and equipment as are made available for the Study under paragraph 2 above.

10. This indemnity shall be governed by and construed in accordance with English law.

SIGNED on behalf of the Health Authority

.....
Chief Executive/District General Manager

30/7/02
.....
Dated

SIGNED on behalf of the Sponsor

.....
Thomas I. H. Dubin
Vice President and General Counsel

Dated 7-15-02

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Date
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Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Summons to attend oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to attend oral proceedings arranged in connection with the above-mentioned European patent.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will be public, will take place before the opposition division

on 15.05.14 at 09.15 hrs in Room 1662 at the EPO, Grasserstr. 2, PschorrHöfe, D-80335 München

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 1/2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to the Special edition No. 3 OJ EPO 2007, 128, concerning the filing of authorisations for company employees and lawyers acting as representatives before the EPO.

The final date for making written submissions and/or amendments (R. 116 EPC) is 17.03.14.

You are requested to report in good time beforehand to the porter in the EPO foyer. Room 1601 and 1657 are available as waiting rooms.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at the porters' lodge in the main foyer "Bayerstrasse", the parking ticket will be revoked.

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

For the Opposition Division

Annexes:
Confirmation of receipt (Form 2936)
Rule 4 EPC (EPC Form 2043)
Communication (EPO Form 2906)

Registered letter with advice of delivery
EPO Form 2310 12.12 [ORAL03=1662] (10/01/14)

ORAL4

to EPO postal service: 10.01.14

CC20871

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be

carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed.

1.6 The P requested oral proceedings in case their respective request would not be granted.

2 Preliminary, non-binding opinion of the opposition division (OD)

The admissibility of the opposition has not been contested by the P. The opposition is thus considered admissible (Article 99(1) and Rule 76 EPC).

The O submitted documents and arguments that would appear to be *prima facie* prejudicial to the maintenance of the patent as granted. Since the O has withdrawn his opposition, the OD feels it pertinent to continue opposition *proceedings upon its own volition* (GL D, VII-5.2,5.3; Article 114(1) EPC).

Added subject-matter (Articles 100(c) and 123(2) EPC)

2.1 The O objected to claims 1 and 5 of the patent on grounds that they are not directly and unambiguously disclosed in the application, rather an intermediate generalisation of a specific example (p.32-33) combined with general teachings of the application.

2.2 The O considered the claims to contain the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter
7. a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to points 4 - 6
8. administration of 900mg every 12 days thereafter

2.3 Features 1 and 2 would appear to have a general basis on p.12, li.1-3 and p. 18, li.12-20, respectively. The Examples on p.33 also contain these features.

2.4 Feature 3 would appear to be broader than the basis provided on p.20, li.4-8, which relates merely to 'intravenous infusion by injection'. Feature 4 would also encompass both injection into a vein using a needle and the use of a catheter. The OD finds that since p.20, li.4-8 neither specifically states the use of a needle or a catheter, 'intravenous infusion by injection' could be considered to encompass both I.V. infusion via needle or catheter, i.e. intravenous administration; however, 'I.V. administration' does not necessarily imply administration of an infusion. 'Infusion' implies the use of a catheter, but this is not stated in the application. The OD is thus of the opinion that **Feature 3 has no basis** in the application as filed as it also encompasses administration of pharmaceutical formulations other than infusions.

2.5 Features 4 - 7 relate to a dosage schedule. The general teaching of the application on p.19-20 states that the dose will vary according to the compound used, the manner of administration, the symptoms, the size and the age of the patient. The only basis for the dosage regimen in the claim comes from the specific example on p.32-33, which describes steps 1 - 7 applied to a patient group. Two of the patients in that group exhibited breakthrough in serum hemolytic activity which was controlled by reducing the dosage interval to 12 days (p.35, li.8-17). The O is of the opinion that the combination of Features 1 - 8 cannot be derived from either the general part of the description or the examples.

2.6 The OD agrees that the only basis for claims 1 and 5 is in the specific examples.

2.7 However, the O argues that 'essential features' of the example are missing from the claim. The OD notes that clarity is not a grounds of opposition. There is no indication in the application to suggest that the specific patient sub-group to be treated must be transfusion-dependent and hemolytic (p.32, li.7-9), that an infusion time of 'approximately 30 mins' (p.30, li.12-14), or that the time point of breakthrough occurrence (between weeks 12-64) are essential to the application claimed. The OD finds that it is clear from the examples that any patient receiving the claimed dosage regimen for PNH and experiencing breakthrough could be treated by shortening the interval between doses, as claimed. The OD does not see that there is an intermediate generalisation of the examples in this respect.

2.8 The O further argues that there is no basis for the route of administration used following the initial four week dosing schedule, i.e. Feature 4, since the route thereafter is not specified. The OD finds that it would be obvious that further antibody treatments would be administered by the same route, however, the O is correct in that this is not clearly stated in the application. This point will require further discussion at oral proceedings.

Lack of novelty (Articles 100(a) and 54 EPC)

2.9 The O raised an objection on grounds of lack of novelty regarding claims 1 and 5 with regard to D1.

2.10 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.11 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.12 The O considered the priority document not to disclose the subject-matter of the present claims for the same reasons they consider the patent's claims not to have a basis in the application as filed (Article 123(2) EPC).

2.13 Since doubt has been raised as to whether a basis for claims 1 and 5 exists in the application, the same doubts result for the disclosure of the priority document. Therefore, the subject-matter of D1 is considered relevant for the assessment of the novelty and inventive step of the claims.

2.14 The OD is of the provisional opinion that claims 1-8 of the contested patent lack novelty over D1.

Lack of inventive step

2.15 The O objected to the patent on grounds of a lack of inventive step, taking D2, D3,D4 or D6 as the closest prior art to the subject matter of claims 1 and 5.

2.16 Documents D3,D4 and D6 all disclose the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks

5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.17 Document D2 does not disclose the Feature 3 and thus will not be considered further.

2.18 The difference between either D3,D4 or D6 and the present claims is considered to be the dosage.

2.19 The technical effect associated with this difference is the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.20 The problem to be solved can therefore be considered to be the provision of an improved dosage regimen for patients suffering a breakthrough in hemolytic activity.

2.21 The solution provided in the granted claims would appear obvious, since according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D3,D4 or D6 were known to be preventable by a slight decrease in the dosing interval.

2.22 The skilled person with a knowledge of any of D3,D4 or D6, and observing a breakthrough in hemolytic activity in patients would therefore find it obvious to slightly reduce the dosing interval until no further hemoglobinuria event is observed (as shown in Fig.5 of the patent).

2.23 The establishment of optimal dosage regimens is considered to be routine according to the case law of the Technical Board of Appeal T1979/09, wherin under point 7.3.1 it is stated *'It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses.'*

2.24 and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step.'*

2.25 It is therefore the provisional opinion of the OD that at least the subject-matter of claims 1 and 5 does not involve an inventive step, in light of either D5 alone, or in combination with any one of D3,D4 or D6.

2.26 Dependent claims 2-4 and 6-8 do not appear to contain any additional features which, in combination with any other claim to which they refer, would meet the requirements of the EPC with respect to inventive step.

Insufficiency of disclosure (Articles 100(b) and 83 EPC)

2.27 The O raised the objection that the trade name 'eculizumab' did not sufficiently disclose such an antibody, since, at the time of filing, such an antibody was not completely described.

2.28 The application makes reference to D9 and D10, which do not make reference to deposits or sequences that disclose the humanised anti-C5 antibody to which it allegedly refers.

2.29 The OD is of the opinion, that in the absence of evidence that eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of* its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed.

2.30 The O made further objections to the clarity of the term 'breakthrough in serum hemolytic activity'. The OD will not pursue this objection since a lack of *clarity is not a ground for opposition.*

Conclusion

3 At present the OD is of the preliminary opinion that the patent contains added subject-matter (Articles 100(c) and 123(2) EPC), does not involve an inventive step (Articles 100(a) and 56 EPC), and lacks sufficient disclosure (Article 100 (b) and 83 EPC).

In view of the request of the P, oral proceedings are hereby appointed.

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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EPO - Munich
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17. März 2014

European Patent Office

80298 MUNICH

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 17, 2014
WA/ARO/FLG

This is in response to the Summons to attend oral proceedings issued on January 14, 2014 and the Brief Communication issued on February 18, 2014 by the European Patent Office in the above-referenced opposition proceedings.

We enclose a new main request and a new auxiliary request which replace the claims as granted of the opposed patent EP-B1 1720571. As it will be evident from the following, both new main requests fulfill the requirements of the EPC. In case patentability of the main request cannot be accepted, consideration of the auxiliary request is requested.

1. AMENDMENTS TO THE CLAIMS (Rule 137(4) EPC)

1.1. Main Request

1.1.1. New claim 1 is based on claim 1 as granted. New claim 5 is based on claim 5 as granted. The term "intravenously" has been replaced by the phrase "by intravenous infusion". This amendment is supported at page 32, line 13 of the

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application as filed. Further support can be found at page 20, line 6 of the application as filed.

1.1.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 as granted.

1.2. Auxiliary Request

1.2.1. New claim 1 is based on claim 1 of the main request. New claim 5 is based on claim 5 as granted. The phrase "wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)" has been added. This amendment is supported by page 38, lines 11-17 and Figure 8 of the application as filed.

1.2.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 of the main request.

2. INADMISSIBLE BROADENING (Articles 100(c) and 123(2) EPC)

2.1. The feature "Intravenous administration" of claim 1

2.1.1. The Opposition Division is of the preliminary view that the feature "intravenous administration" of claim 1 (i.e. feature 3 as characterized by the Opposition Division) extends beyond the contents of the application as filed, because it may also encompass administration by means other than infusions.

2.1.2. New claims 1 and 5 of the main request have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The same amendment is present in claims 1 and 5 of the auxiliary request. The phrase "by intravenous infusion" has a clear and unambiguous basis at page 32, line 13 and page 20, line 6 of the application as filed.

We therefore submit that new claims 1 and 5 of the main request as well as auxiliary request comply with Article 123(2) EPC.

2.2. Mode of administration of the dosage schedule

2.2.1. The Opponent has argued that there is no basis in the application as filed that the dosages following the administration of 600mg eculizumab each week for 4 weeks are administered by the same route. The Opposition Division states that it is obvious that also the remainder of the administration steps are administered by the same route. However, since the Opponent's observation that no

administration route is expressly mentioned in connection with the further administration steps is correct, the Opposition Division wishes to further discuss this point at the oral proceedings.

2.2.2. In preparation of the discussion of this issue at the oral proceedings, we wish to provide the following comments in writing. We fully concur with the Opposition Division's statement that it is obvious that also the further administration steps are administered by the same route. The disputed passage at page 32 of the application reads:

> "Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis. The first twelve weeks of the study constituted the pilot study." (emphasis added)

Nothing in this passage conveys the impression that the mode of administration via intravenous infusion is changed after the initial dosing step of 600mg eculizumab per week for four weeks. The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered by intravenous infusion makes no sense. In fact, if the mode of administration were to be changed, a corresponding statement would be expected in the corresponding passages. This is, however, not the case. The only mode referred to in the examples is intravenous infusion.

In fact, the attitude of the skilled person is to read the contents of a patent specification with a mind willing to understand the technical sense, not a mind desirous of misunderstanding (T 190/99; confirmed *inter alia* in T 1204/06 or T 1771/06). Although this case law on how a skilled person reads the contents of an application has been established in connection with Article 84 EPC, the Boards have established essentially the same standard under Article 123(2) EPC. In this regard reference is made to T 609/95, reasons for the decision 2.3:

> "The board takes the position that where a drafting defect or inconsistency in an application would be evident to a reader skilled in the art, the person to whom the application is addressed, it is reasonable to suppose that he would, in the light of the content of the application, attempt to formulate a notional amendment which would enable him to make sense of what he reads, and to the extent that the amendment might be said to leap to the mind of the reader, although perhaps only after close study of the document, it can be regarded as implicit in the application and would not contravene Article 123(2) EPC, if effected in practice." (emphasis added)

Samsung Bioepis Exhibit No. 1028
Page 513

Such standard applies all the more to the present case, given that there is no drafting defect or inconsistency. The principle that the skilled person tries to sensibly interpret the contents of the application also with regard to claim amendments has been confirmed in T 887/97, reasons for the decision 2.2. Hence, also when it comes to the assessment of the contents of the application under Article 123(2) EPC, the skilled person aims at making sense of what he reads in an application.

It is furthermore emphasized that the application is addressed to the skilled person and hence the contents of the application as filed have to be assessed from the perspective of the skilled person. The skilled person unequivocally reads the contents of the above-quoted passage of the application as filed in the light of the available knowledge at the priority date of the opposed patent.

In view of the above-recited case law, the skilled person cannot understand the above-quoted passage from the application other than that eculizumab is administered by intravenous infusion throughout the entire study, especially in view of the fact that no different mode of administration is mentioned in the Examples.

2.3. In summary we submit that the new claims of the main and auxiliary request have a clear and unambiguous basis in the application as originally filed.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opposition Division is of the preliminary view that feature 3 of granted claim 1 and arguably also the mode of administration of the dosage schedule have no proper basis in the priority application. For this reason, the Opposition Division is of the view that the claims are no entitled to priority and consequently D1 becomes prior art.

The Opposition Division states that D1 anticipates claims 1 to 8.

3.2. The feature "Intravenous administration" of claim 1

The new claims have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The skilled person can clearly and unambiguously derive the mode of "intravenous infusion" from page 30, line 14 and page 19, line 17 of the priority-establishing application.

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3.3. Mode of administration of the dosage schedule

The passage at page 32, lines 12-15 of the application as filed corresponds to the contents of the priority-establishing application at page 30, lines 13-19.

In G 1/03, G 2/03 and G 2/10 the Enlarged Board held that the disclosure as the basis for the right to priority under Article 87(1) EPC, and as the basis for amendments in an application under Article 123(2) EPC have to be interpreted in the same way. Hence, for the same reasons set forth in section 2.2.2, *supra* it is immediately evident to the skilled person – in the sense that nothing else can be meant - that eculizumab is administered by intravenous infusion in the entire study as described in the priority-establishing application.

3.4. With regard to the auxiliary request it is to be added that the phrase "wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)" can be clearly and unambiguously derived from page 34, lines 13-19 and Figure 8 of the priority document.

3.5. We therefore submit that the skilled person can immediately derive the subject-matter of the new main and auxiliary request from the priority-establishing application. The time rank of the new claims is consequently February 3, 2004; i.e. before D1 became available to the public. D1 is not prior art for the new set of claims.

4. INVENTIVE STEP (Articles 100(a) and 56 EPC)

4.1. The Opposition Division is of the preliminary view that at least claims 1 and 5 lack an inventive step in view of D5 optionally combined with D3, D4 or D6.

The Opposition Division defines the technical problem underlying the claimed subject-matter as the provision of an improved dosage regimen of eculizumab.

The solution of this technical problem - i.e. the administration of 900mg eculizumab every 12 days instead of every 14 days – is allegedly obvious, because D5 teaches that transient complement breakthrough can be prevented by a slight decrease in dosing interval.

The Opposition Division thus takes the position that a skilled person starting from D5 would have reduced the dosing interval until no complement

breakthrough is observed. By referring to decision T 1979/09, reasons for the decision sections 7.3.1 and 7.3.2, the Opposition Division emphasizes that finding the optimal dosage interval cannot justify the presence of an inventive step.

4.2. Our arguments indicating the presence of an inventive step submitted on December 31, 2013 are fully maintained and still applicable to the enclosed new main and auxiliary requests.

In order to underline our position that the skilled person starting from D5, optionally combined with D3, D4 or D6, would not have arrived at the exact dose of 900 mg eculizumab every 12 days without the need of inventive skills, the following further arguments are provided.

4.2.1. D5 teaches:

> "Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms; these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval." (emphasis added)

For a proper assessment of inventive step, we would like to elaborate on the following issues: What would the skilled person have observed upon slightly decreasing the interval, in particular from 14 days to 13 days? Would he/she have been encouraged to further decrease the interval?

4.2.2. The attention of the Opposition Division is respectfully drawn to Figure 4 of the application as filed. Figure 4 shows hemolysis tests on urine samples of the studied PNH patients. In more detail, in the urine samples the levels of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured. LDH and AST are often used in the art as a marker of hemolysis, as LDH and AST are abundant in red blood cells. Upon red blood cell lysis LDH and AST are released from the cell to the extracellular compartments of the body. Consequently, LDH and AST can be detected in a urine sample if red blood cell lysis occurs. In the absence of red blood cell lysis LDH and AST cannot be detected in a urine sample. It follows that the more LDH or AST is found in the urine sample, the more hemolysis occurs.

The pharmacokinetics (PK) and pharmacodynamics (PD) of eculizumab were also measured. PK is a measure of the concentration of eculizumab in the serum of a patient over time and PD is a measure of the level of complement activity in the patient's serum. The method used for the determination of PD levels is described at page 37, line 10 to page 38, line 10 of the application as filed. High PD levels indicate high levels of complement activity in the patient's serum, which in turn correlates with high levels of hemolysis.

The urine samples tested in Figure 4 have been obtained from PNH patients who received a dose of 900mg eculizumab every 14 days. The day on which 900mg eculizumab was administered is characterized as day "0" in Figure 4. Urine samples were obtained at day "0" and each day between day "9" and day "13" of the thirteen days between the mentioned fortnightly administrations of 900mg eculizumab.

At day 0, i.e. before the administration of eculizumab, LDH, AST and PD levels were consistently high, thereby indicating the occurrence of hemolysis in the tested PNH patients. LDH, AST and PD levels again were measured on days 9, 12 and 13 after administration of eculizumab. The measured LDH, AST, and PD consistently show that no hemolysis was found until day 12. However, on day 13, LDH, AST and PD levels surprisingly increased dramatically, thereby indicating an abrupt relapse of hemolytic activity in the PNH patients. In particular, the PD levels as shown in Figure 4 demonstrate the dramatic increase of serum hemolytic activity from day 12 to day 13:

	Day 12	Day 13	Day 14 = day 0[1]
PD	2	56	56

(excerpt of contents of Figure 4 of the application as filed)

Hence, the data in Figure 4 of the application as filed evidence that the administration of 900mg eculizumab every 12 days – as claimed – is the optimum dosage regimen for the administration of eculizumab. Assuming that 900mg eculizumab would be administered every 13 days, complement

[1] We note that day 14 is designated day 0 in Figure 14, given that the next dose is administered.

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breakthrough may be still observed in PNH patients. If 900mg eculizumab were administered every 11 days, this would be an unnecessarily short interval which is of no further benefit for the PNH patients. Hence, Figure 4 evidences that the optimal time window for the administration of 900mg eculizumab is very narrow. In fact, the claimed 12 days dosing interval is ideal, while administration every 11 or 13 days cannot be viewed as ideal dosing intervals.

Importantly, the data in Figure 4 evidence that a decrease from 14 to 13 days is not encouraging. The breakthrough patients would remain in the realm of high LDH and unfavorable PD.

4.3. Since the Opposition Division relies on T 1979/09, reasons for the decision 7.3.1 and 7.3.2, for concluding that finding an optimal dosage interval may justify the presence of an inventive step, we will demonstrate in the following that the case decided in T 1979/09 has to be held distinct from the present situation.

4.3.1. *Dosage range vs. exact dosage*

It can be taken from reason for the decision 7.1 that the claims at stake in T 1979/09 did not refer to an exact dosage but to a broad dosage range of 300mg to 800mg of the extract in 1 to 3 capsules of tablets taken once daily. Thus, irrespective of whether 300mg of the extract or 800mg of the extract are administered as daily treatment, success is ensured.

On the other hand, claim 1 of the enclosed new main and auxiliary request refers to a distinct dose of 900mg eculizumab at a distinct time interval of every 12 days. As it has been detailed in section 4.2, *supra*, the precisely and narrowly defined dosing scheme according to step (iv) of claim 1 of the enclosed new main and auxiliary request – being the administration of 900mg eculizumab every 12 days – ensures successful treatment of PNH breakthrough patients. As such the facts of the case underlying in T 1979/09 on the one hand and the present case on the other hand are fundamentally different. It was by no means obvious from D5 optionally combined with D3, D4 or D6 for the skilled person to arrive at the mentioned narrow and specific dosing schedule.

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4.3.2. *Closest prior art in T 1979/09 and in the present case*

We consider it also important to note that the prior art in T 1979/09 was considerably closer to the claimed subject-matter as in the present case. As it is evident from reason for the decision 7.3, the prior art taught to administer a total daily dose of 360mg to 540mg (which is within the claimed range of 300mg to 800mg) of the extract in 2 to 3 capsules (which is within the claimed range of 1 to 3 capsules) twice a day. It follows that in T 1979/09 the claimed total daily dose of the extract comprised that of the prior art. The only difference is that the dose is administered once a day instead of twice.

In the present case D5 may be seen as the closest prior art document. D5 merely mentions a "slight decrease in dosing interval" but fails to suggest any specific interval. Hence, the gap between D5 and the claimed subject-matter is indeed wider than the gap between the closest prior art and the claimed subject-matter in the case at issue in T 1979/09. The skilled person was not able to close the gap in the present situation without the need of inventive skills. D5 conveys the impression that a slight decrease of the dosing interval of 14 days would prevent complement breakthrough. However, as set forth in section 4.2 of this petition a decrease from 14 days to 13 days – which certainly is much closer to a "slight decrease" than from 14 to 12 days - fails to prevent further complement breakthroughs. As detailed herein above, administering 900mg eculizumab at an interval of every 12 days ensures optimal treatment.

This was by no means foreseeable for the skilled person on the basis of D5 optionally combined with D3, D4 or D6. None of these documents guides the skilled person to the interval of 12 days.

4.3.3. *Presence of an unexpected technical effect*

Section 7.3.2 of the Reasons for the decision of T 1979/09 reads:

> "In the present case the amount of active ingredient remains the same as the amount used in D5 [i.e. the closest prior art document in T 1979/09] and there is no evidence on file of any unexpected technical effect caused

> by administering the red vine leave extract once daily." (information in brackets and emphasis added)

Hence, in T 1979/09 no technical advantage associated with the distinguishing feature from the closest prior art could be demonstrated.

Moreover, section 7.3.3 of the Reasons for the decision of T 1979/09 teaches:

> "It has never been shown that taking a dose of 300-800 mg once daily has the same effect as taking the same dose twice daily."

As such, it even has not been shown in the case at issue in T 1979/09 that administering the extract once a day is as beneficial as twice a day.

On the other hand, in the present application it has been demonstrated that unexpected technical effects are associated with administering 900mg eculizumab every 12 days. The interval of 12 days is the optimum while shorter as well as longer intervals are associated with the discussed drawbacks. It is our firm belief that the advantageous technical effects being associated with an interval of 12 days fully justify the acknowledgment of an inventive step.

4.4. For the sake of completeness we note with regard to the auxiliary request that the feature of "reducing cell lysis to below 20% of normal serum complement activity" is a feature indicating the effective inhibition of haemolytic activity; see page 38, lines 11-13 and Figure 8. The effective inhibition of haemolytic activity is a key advantage being associated with the subject-matter claimed in the main and auxiliary requests. This advantage is expressly incorporated in the main claim of the auxiliary request.

4.5. In summary, we submit that the enclosed main and auxiliary requests meet the requirements of Article 56 EPC.

5. SUMMARY AND REQUESTS

In view of the above, we submit that the set of claims constituting the main request meets the requirements of the EPC. In case the patentability of the

main request cannot be acknowledged consideration of the auxiliary request is requested.

We further request that in view of our submissions, oral proceedings are cancelled and the proceedings be continued in writing. In case the Opposition Division comes to a different conclusion, we request that we be allowed to submit one or more additional claim request(s) on the occasion of the oral proceedings.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
New main and auxiliary requests

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L2 ANSWER 1 OF 1 REGISTRY COPYRIGHT 2018 ACS on STN
RN 219685-50-4 REGISTRY
CN Immunoglobulin, anti-(human complement C5 α -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
SQL 1324,448,448,214,214
NTE multichain

type	------ location ------		description
bridge	Cys-22	- Cys-96	disulfide bridge
bridge	Cys-136	- Cys-214''	disulfide bridge
bridge	Cys-149	- Cys-205	disulfide bridge
bridge	Cys-228	- Cys-228'	disulfide bridge
bridge	Cys-231	- Cys-231'	disulfide bridge
bridge	Cys-262	- Cys-322	disulfide bridge
bridge	Cys-368	- Cys-426	disulfide bridge
bridge	Cys-22'	- Cys-96'	disulfide bridge
bridge	Cys-136'	- Cys-214'''	disulfide bridge
bridge	Cys-149'	- Cys-205'	disulfide bridge
bridge	Cys-262'	- Cys-322'	disulfide bridge
bridge	Cys-368'	- Cys-426'	disulfide bridge
bridge	Cys-23''	- Cys-88''	disulfide bridge
bridge	Cys-134''	- Cys-194''	disulfide bridge
bridge	Cys-23'''	- Cys-88'''	disulfide bridge
bridge	Cys-134'''	- Cys-194'''	disulfide bridge
uncommon	Glp-1	-	-
uncommon	Glp-1'	-	-

```
SEQ      1 XVQLVQSGAE VKKPGASVKV SCKASGYIFS NYWIQWVRQA PGQGLEWMGE
        51 ILPGSGSTEY TENFKDRVTM TRDTSTSTVY MELSSLRSED TAVYYCARYF
       101 FGSSPNWYFD VWGQGTLVTV SSASTKGPSV FPLAPCSRST SESTAALGCL
       151 VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSNFGT
       201 QTYTCNVDHK PSNTKVDKTV ERKCCVECPP CPAPPVAGPS VFLFPPKPKD
       251 TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST
       301 YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY
       351 TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD
       401 SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK

SEQ      1 XVQLVQSGAE VKKPGASVKV SCKASGYIFS NYWIQWVRQA PGQGLEWMGE
        51 ILPGSGSTEY TENFKDRVTM TRDTSTSTVY MELSSLRSED TAVYYCARYF
       101 FGSSPNWYFD VWGQGTLVTV SSASTKGPSV FPLAPCSRST SESTAALGCL
       151 VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSNFGT
       201 QTYTCNVDHK PSNTKVDKTV ERKCCVECPP CPAPPVAGPS VFLFPPKPKD
       251 TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST
       301 YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY
       351 TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD
       401 SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK

SEQ      1 DIQMTQSPSS LSASVGDRVT ITCGASENIY GALNWYQQKP GKAPKLLIYG
        51 ATNLADGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQN VLNTPLTFGQ
       101 GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
       151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
       201 LSSPVTKSFN RGEC

SEQ      1 DIQMTQSPSS LSASVGDRVT ITCGASENIY GALNWYQQKP GKAPKLLIYG
        51 ATNLADGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQN VLNTPLTFGQ
```

L = site of inserted stretches of amino acids

O = substituted amino acids

— = missing amino acids

101 GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN RGEC

MF Unspecified
CI MAN
SR US Adopted Names Council (USAN)
LC STN Files: ADISINSIGHT, BIOSIS, CA, CAPLUS, CHEMCATS, CHEMLIST, EMBASE, IMSRESEARCH, IPA, MEDLINE, PATDPASPC, RTECS*, TOXCENTER, USPAT2, USPATFULL
(*File contains numerically searchable property data)
DT.CA CAplus document type: Conference; Dissertation; Journal; Patent
RL.P Roles from patents: ANST (Analytical study); BIOL (Biological study); NANO (Nanomaterial); PREP (Preparation); PROC (Process); PRP (Properties); PRPH (Prophetic); RACT (Reactant or reagent); USES (Uses)
RLD.P Roles for non-specific derivatives from patents: ANST (Analytical study); BIOL (Biological study); PREP (Preparation); PRP (Properties); USES (Uses)
RL.NP Roles from non-patents: ANST (Analytical study); BIOL (Biological study); OCCU (Occurrence); PREP (Preparation); PROC (Process); PRP (Properties); USES (Uses)
753 REFERENCES IN FILE CA (1907 TO DATE)
23 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
788 REFERENCES IN FILE CAPLUS (1907 TO DATE)

Experimental Report - Increased Plasma Half-Life

Methods

The pharmacokinetics of eculizumab (n=5) and the anti-C5 IgG4 monoclonal antibody described in Thomas et al. (1997) Mol. Immunol. 33: 1389-1401) (n=3) were compared using FcRn transgenic mice strain 32 that harbor a knockout allele of the FcRn α-chain and express a human FcRn α-chain (FCGRT) transgene under control of the human FcRn promoter. (Jackson Labs https://www.jax.org/strain/014565).

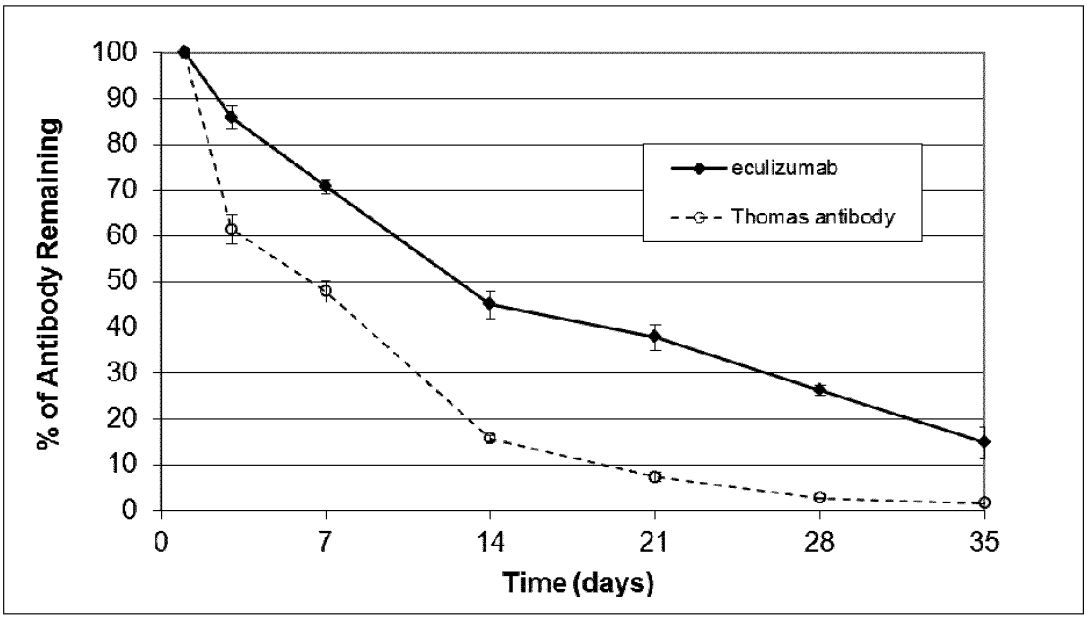
100µg of each antibody in 200µl of PBS was administered intravenously via the tail vein to female human FcRn transgenic mice at time zero. At 3, 7, 14, 21, 28 and 35 days following administration of each mAb, samples (100 µl) of blood was taken via retro-orbital bleeding and assayed for the levels of human antibody using an ELISA in which human antibody was captured with sheep anti-human kappa antibody and detected with HRP-conjugated sheep anti-human IgGg as follows: 96 well ELISA plates were coated with 1:1000 dilution of an anti-human Kappa antibody, 1:1000 (AU015, The Binding Site). Samples of mouse serum containing the humanized antibodies and standards, each diluted in mouse serum, were then applied to the plates at a final serum dilution of 1:1600. Following washing, the bound humanized antibody was probed with an HRP-conjugated anti-human IgG (gamma) antibody at a 1:1000 dilution (AP004, The Binding Site). Plates were incubated for 30 min and read at A450nm. Analysis of absorbance data was with Softmax Pro software incorporating a 4 parameter fit. Half-life values (t1/2) were calculated using the following equation:

$t1/2 = T \times Ln(2)/Ln(A0/At)$

Where T = time in hr.
Ln(2) = natural log of 2
A0 = absorbance of sample at time 0
At = absorbance of sample at time t"

Results

The Figure below shows the percentage of antibody remaining in the serum over time measured using an ELISA format. Eculizumab has different pharmacokinetics to the IgG4 subtype antibody (Thomas antibody) and is cleared from the serum more slowly. The half-life (t1/2) for eculizumab is 12.56 ± 1.73, whilst the half-life (t1/2) for the IgG4 subtype antibody (Thomas antibody) is 5.95 ± 0.18.


% of Antibody Remaining
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
Time (days)
eculizumab
Thomas antibody

Definitions of Eculizumab in patents and applications cited in D8

Publication No.	Title	Reference to D7/D11	Status
EP 2380907 B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	"These antibodies disclosed in U.S. Patent 6,355,245 include a preferred whole antibody (now named eculizumab)." - paragraph [0043]	Granted - not opposed
EP 1988882 B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	"These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab)" - paragraph [0086]	Revoked
EP 1755674 B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	"These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab)" - paragraph [0055]	Revoked and appeal filed - claims no longer recite eculizumab
EP 2067810 B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	"These antibodies disclosed in U.S. Patent 6,355,245 include a preferred whole antibody (now named eculizumab)." - paragraph [0044]	Granted - not opposed
EP 1720571 B9	METHOD OF TREATING HEMOLYTIC DISEASE	"Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab." - paragraph [0040]	Revoked
EP 3056218 A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	"These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named	Refused and appeal filed - latest claims do not recite eculizumab

		pexelizumab).” – paragraph [0055]	
EP 2910253 A1	METHOD OF TREATING HEMOLYTIC DISEASE	“Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and “Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv”, Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.” – paragraph [0040]	Withdrawn
EP 2815767 A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	“These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab).” – paragraph [0055]	Withdrawn
EP 2089058 A2 (WO 2008/069889)	METHODS OF TREATING HEMOLYTIC ANEMIA	“Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and “Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv”, Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.” – page 19, line 30 – page 20, line 3	Withdrawn

EP 2013235 A1 (WO 2007/130031)	METHODS FOR THE TREATMENT OF MUSCULUAR DYSTROPHY ASSOCIATED WITH DYSFERLIN-DEFICIENCY	"These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab)." – page 12, lines 13 to 16	Withdrawn
EP 1881993 A2 (WO2006/122257)	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES	"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… Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic." – page 2, lines 7-9 and 12-14	Withdrawn

Cited Documents

D1 US 2005/191298

D2 Hillmen et al. (2003)

D3 Hillmen et al. (2004)

D4 Harding (2004)

D5 Hill (2005)

D6 CAS database registry

D7 Thomas et al. (1996)

D8 Table of Alexion European patents and patent applications referring to "eculizumab"

D9 Submission of Alexion dated 30 December 2013 in opposition proceedings against EP 1720571

D10 WO 2005/074607

D11 US 6355245

D12 Bell declaration (filed 10 July 2017)

D13 Boone declaration (filed 10 July 2017)

D14 Andrien declaration (filed 10 July 2017)

D15 Letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007 (filed 16 January 2018)

D16 Press release made by Alexion Pharmaceuticals on 16 March 2007 (filed 16 January 2018)

D17 Saxe declaration (filed 16 January 2018)

D18 Experimental report (filed 16 January 2018)

D19 Second Andrien declaration (filed 15 August 2018)

D20 Extract from Alberts et al. (1994) (filed 15 August 2018)

D21 Opposition statement filed by Pfizer against EP 1720571 (filed 15 August 2018)

D22 Redacted version of clinical trial protocol C04-001 (filed 15 August 2018)

D23 Redacted version of clinical trial protocol C02-002 (filed 15 August 2018)

D24 Table of Alexion patents and patent applications showing how these reference D7 and D11, and providing the status of each case

D25 Summons and preliminary opinion of Opposition Division in opposition against EP 1720571

D26 Response filed by Alexion on 17 March 2014 to summons and preliminary opinion in opposition against EP 1720571

D27 Decision revoking EP 1720571

D28 Annotated version of original CAS Registry No. 219685-50-4 obtained from http://commonchemistry.org/ showing the errors in the sequences

D28a Annotated version of corrected CAS Registry No. 219685-50-4 obtained from CAS

D29 Second Experimental Report

D30 Wang et al. (2007) J. Pharmaceutical Sciences 96(1): 1-26

D31 Bombara declaration

AUXILIARY ~~MAIN~~ REQUEST 2
CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria~~the antibody of claim 1~~.

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AUXILIARY REQUEST 1
CLAIMS

1. An antibody that binds C5 comprising the ~~a~~ heavy chain amino acid sequence shown in ~~consisting of~~ SEQ ID NO: 2 and the~~a~~ light chain amino acid sequence shown in ~~consisting of residues 23 to 214 of~~ SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

AUXILIARY REQUEST ~~1~~3
CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

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Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	7641517
Application number	EP16195355.9
Date of receipt	05 July 2019
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file

Documents submitted

package-data.xml	ep-sfd-request.xml
epf1038.pdf (2 p.)	ORAL-1.PDF\Final Written Submissions N411599EP-B PXT.PDF (18 p.)
CLMS-1.PDF\Auxiliary Request 1 (clean) N411599EP-B PXT.PDF (1 p.)	CLMS-HWA-1.PDF\Auxiliary Request 1 (tracked) N411599EP-B PXT.PDF (1 p.)
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ORAL-4.PDF\D25 Summons to OPs on EP1720571 - N411599EP-B.PDF (7 p.)	ORAL-5.PDF\D26 Alexion response to Summons on EP1720571 - N411599EP-B.PDF (11 p.)
ORAL-6.PDF\D27 Decision revoking EP1720571 - N411599EP-B.PDF (11 p.)	ORAL-7.pdf\D28 - Annotated CAS Registry.pdf (2 p.)

ORAL-8.pdf\D28A - Annotated CAS Registry.pdf (2 p.) ORAL-10.pdf\D30 - Wang et al..pdf (26 p.)	ORAL-9.PDF\D29 Experimental report N411599EP-B.PDF (2 p.) ORAL-11.PDF\D31 Bombara Declaration.PDF (90 p.)

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Correction by the EPO of errors in debit instructions filed by eOLF
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Date: 18.07.2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Oral Proceedings on 05.09.19 at 09:00 hours

Subject:
- ☒ Your letter of ...05.07.2019.....
- ☐ Maintenance / Cancellation / postponement at the instigation of the division
- ☐ Consultation by telephone on.....(EPO Form 2036)

Communication:

1. ☒ The date / time fixed for oral proceedings is maintained.
   - ☐ The reasons are indicated on enclosed EPO Form 2906.
2. ☐ The above-mentioned oral proceedings will start at ..... hours.
3. ☐ The summons to attend oral proceedings on the date indicated above is cancelled.
   - 3.1 ☐ The reasons are indicated on enclosed EPO Form 2906.
     - ☐ The procedure will be continued in writing.
     - ☐ A new date will be set later.
     - ☐ New summons will follow.
   - 3.2 ☐ For administrative reasons the oral proceedings are postponed to a later date. New summons will follow.
   - 3.3 ☐ The application is deemed to be withdrawn. The right to oral proceedings only persists as long as proceedings are pending.
4. ☐ The request for the oral proceedings to be held by videoconference:
   - 4.1 ☐ is allowed.
   - 4.2 ☐ is rejected.
     - ☐ No videoconference studio is available on the date indicated above.
     - ☐ See enclosed EPO Form 2906.

Registered letter
EPO Form 2008A 01.19 (11/07/19)

SS51521

Samsung Bioepis Exhibit No. 1028
Page 536

5. ☒ Please confirm receipt of this communication by return fax with date stamp and signature to the EPO central fax server in Munich: (+49) (0) 89 23 99 44 65.

Please take note.

For the Examining Division

Enclosure(s): ☐ EPO Form 2906

☐ EPO Form 2036

Samsung Bioepis Exhibit No. 1028
Page 537

The examination is being carried out on the **following application documents**

Description, Pages

1-55 as originally filed

Sequence listings, SEQ ID NO

1-10 as originally filed

Claims, Numbers

1-21 as originally filed

Drawings, Sheets

1/4-4/4 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01)

D2 HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)",
BRITISH JOURNAL OF HAEMATOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB,

vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758,
ISSN: 0007-1048

D3 HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA",
NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US,
vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948,
ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688

D4 HARDING J: "Eculizumab",
DRUGS OF THE FUTURE, PROUS SCIENCE, ES,
vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000,
ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330

D5 US 2005/221382 A1 (ROTHER RUSSELL P [US]) 6 October 2005 (2005-10-06)

D6 HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria",
CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US,
vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426,
ISSN: 1543-0790

For what concerns the most relevant passages of the above-mentioned documents , please see citations in the European Search Report, unless otherwise stated.

1. Novelty

The present application formally meets the requirements of Article 54(1) and (2) EPC because the subject-matter of the claims is not exactly disclosed by the available prior art documents.

2. Inventive step

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of the whole set of claims does not involve an inventive step within the meaning of Article 56 EPC.

Document D1 may me considered as the closest prior art to the subject-matter of the present application and discloses the use of any compound which binds or otherwise blocks the generation and/or activity of one or more complement component in the treatment of paroxysmal nocturnal hemoglubinuria. In particular, anti-C5 antibodies are described by D1: eculizumanb and pexelizumab.

D1 also discloses the dosage regimen for the described treatment: an aqueous solution comprising eculizumab is administered to the patients in a dosage of 600 mg weekly for four weeks, followed by a 900 mg eculizumab one week after, and 900 mg eculizuman bi-weekly till the end of the treatment. The dosage regimen indicated by D1 corresponds exactly to the one claimed in present claims 15 and 19, both referring back to a single unit dosage form of the ant-C5 antibody or C5-binding fragment thereof.

It should be noted by the applicant that the dosage regimen specified in the application and disclosed by D1 is to be considered as the conventionally used one since it is disclosed by many other prior art documents (see for example D2-D4).

The difference between the claimed subject- matter and the disclosure of D1 is therefore nly represented by the indication that the claimed pharmaceutical composition comprises a single unit dosage form.

As a first remark, it should be noted that the present application does not indicated any advantage linked to the formulation of the claimed compounds in a single unit dosage form compared to any other forms disclosed by the prior art.

Moreover, D5 already teaches that aqueous formulations of the disclosed compounds, although used for the treatment of a different disease, may be presented in unit dosage forms and prepared according to conventional techniques well known in the pharmaceutical industry.

Thus, in the absence of any surprising or unexpected technical effect due to the formulation of the claimed compounds in a single unit dosage from, the skilled person would have come to the claimed invention without any exercise of an inventive activity.

The attention of the applicant is also drawn to the fact that a single unit dosage form of the mentioned compounds in an amount of 300 mg, would obviously be the most convenient one since for administering 600 mg of the active, two dosage forms and for administering the 900 mg of the active three of them can be used without any wast of active agent.

3. Further procedure of the application

It is not at present apparent which part of the application could serve as a basis for a new, allowable claim. Should the applicant nevertheless regard some particular matter as patentable, an independent claim should be filed taking account of Rule 43 (1) EPC. The applicant should also indicate how the subject- matter of the new claim differs from the state of the art and the significance thereof.

The attention of the applicant is drawn to the fact that the application may not be amended in such a way that it contains subject- matter which extends beyond the content of the application as filed (Article 123(2) EPC).

The examination is being carried out on the **following application documents**:

Description, Pages

1-39 as published

Claims, Numbers

1-27 filed with entry into the regional phase before the EPO

Drawings, Sheets

1/11-11/11 as published

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1: ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia." HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825 ISSN: 1520-4391

D2: JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough" 19000101, [Online] 13 December 2002 (2002-12-13), pages 1-2, XP009106338 Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle /446336> [retrieved on 2008-09-24]

D3: "Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria" TRANSPLANT NEWS, [Online] 15 January 2004 (2004-01-15), XP002497241 Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_ m0YUG/is_1_14/ai_n17208612> [retrieved on 2008-09-25]

D4: HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)" BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083 ISSN: 0006-4971

D5: THOMAS T C ET AL: "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv" MOLECULAR IMMUNOLOGY, ELMSFORD, NY, US, vol. 33, no. 17-18, 1 January 1996 (1996-01-01), pages 1389-1401, XP002262113 ISSN: 0161-5890

D6: HILLMEN PETER ET AL: "Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria." THE NEW ENGLAND JOURNAL OF MEDICINE 5 FEB 2004, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948 ISSN: 1533-4406

D7: FITCH JC ET AL: 'Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass' CIRCULATION vol. 100, no. 25, December 1999, pages 2499 - 2506, XP002209328

D8: DATABASE PUBMED 16464755 HILL A ET AL: 'Improvemnet in the symptons of smooth muscle dystonia during eculi therapy in paroxysmal nocturnal hemoglobinuria', XP003000273

1.0 Introduction

The present application relates to treatments for the effects of hemolytic disease using an anti-C5 (complement component C5) antibody (h5G1.1; Eculizumab). A small study indicates also that Eculizumab treatment in combination with administration of erythropoietin can reduce transfusion requirements of thrombocytopenic patients, albeit only for those with a good marrow reserve.

2.0 Article 123(2) EPC

The amended claims 1-27 are considered allowable under Article 123(2) EPC.

3.0 Article 84 EPC

3.1 Claims 1-5,8-10,12-14,16 and 17 do not meet the requirements of Article 84 EPC in that the matter for which protection is sought is not clearly defined. The following functional statements do not enable the skilled person to determine which technical features are necessary to perform the stated functions:

"Use of a molecule selected from the group consisting of (a) molecules which bind to one or more complement components, (b) molecules which block the generation of one or more complement components, and (c) molecules which block the activity of one or more complement components for ... "

The suggested grouping is so broad as to include a variety of molecules, of which it is not clear whether all would indeed be suitable for the desired use. For example, there are several known antibodies that can bind complement, but which do not modulate its function. Furthermore, molecules that interfere with members of the complement cascade prior to C5 may create a vulnerability to both pyogenic infections and autoimmune disease and thus would not be suitable (See document D1, Fig 4).

3.2 Paroxysmal nocturnal hemoglobinuria (PNH) is characterised by hemolysis, hemoglobinuria, thrombosis, anemia, erectile dysfunction, esophageal spasm (dysphagia), and abdominal pain (see p2, para.2 of the description). It could therefore be expected that any treatment for PNH should relieve some of these symptoms. However, the formulation of present claims 2-5,8 and 16 implies that the invention is applicable for the treatment of these symptoms in all patients. Present claims 8 and 9, for example, refer to a composition for increasing the proportion of type III red blood cells of a subject`s total red blood cell count, however, it is not clear from the application whether the composition claimed would be effective in all humans or just those with hemolytic disease.

3.3 The definition of the term "hematopoiesis" defined in the description (p7, li. 22-25) and referred to in claims 13,14,26 and 27 shall be taken to be that commonly accepted in the field of hematology i.e. the formation of blood cells in the body. Thus, substances which simply prolong the life of blood cells will not be considered to be included under this term.

3.4 Further medical use claims 1,8,12 and 17 are not acceptable under Article 84 EPC. The therapeutic application is functionally defined by a mechanism of action which does not allow any practical application in the form of a defined, real treatment of a pathological condition (disease) (see Guidelines C-IV, 4.8).

The objection could be overcome by either introducing into the claims a list of

pathological conditions (diseases) cited in the application, or by showing that means are available which would allow the skilled person to recognise which additional condition/s would fall within the functional definition (see Guidelines C-III, 6.5).

3.5 Claims 1-5,8,10,12,13,16 and 17 have been drafted as separate independent claims.

Under Article 84 in combination with Rule 43(2) EPC, an application may contain more than one independent claim in a particular category only if the subject-matter claimed falls within one or more of the exceptional situations set out in paragraph (a), (b) or (c) of Rule 43(2) EPC. This is not the case in the present application, however, for the following reasons: the inventions as claimed are not obviously related.

In the further prosecution of the application, failure to file an amended set of claims which complies with Rule 43(2) EPC, or to submit convincing arguments as to why the current set of claims does in fact comply with these provisions, may lead to refusal of the application under Article 97(2) EPC.

4.0 Article 54 EPC

Furthermore, notwithstanding the above-mentioned lack of clarity, the subject-matter of claims 1-12 and 16-24 is not new within the meaning of Article 54(1) and (2) EPC, and therefore the requirements of Article 52(1) EPC are not met.

4.1 Document D1 discloses that humanised monoclonal antibody against complement C5 has "a remarkable effect in controlling the manifestations of [hemolytic] disease". For example, paroxysmal nocturnal hemoglobinuria (PNH) is characterised by hemolysis, hemoglobinuria, thrombosis, anemia (p48, col.1, last para.), erectile dysfunction, esophageal spasm (dysphagia), and abdominal pain (p49, col.1 lines 31-33). The latter two resulting from reduced NO, since in PNH, released hemoglobin scavenges NO thereby reducing free NO in the blood (p49, col.2, last para). The document describes how the anti-complement C5 mAb,

Eculizumab, blocks the creation of the membrane attack complex, preventing the release of the potent anaphylatoxin C5a and hemolysis (and thus hemoglobin release). Since Eculizumab treatment leads to an increase in the proportion of PNH red cells of 36.7-59.2% (p53, col.1, lines 40-46) patients with hemolytic disease may therefore cease or require less frequent transfusions. This document also mentions the use of soluble, membrane-anchorable CD59 to prevent hemolysis (p53, second para.).

4.2 Document D2 is a report from 13.12.2002 based on Abstract 154 presented on 9.12.2002 at the 44th Annual Meeting of the American Society of Hematology (ASH) and refers to the use of Eculizumab for the treatment of the symptoms of PNH, in particular hemolysis. The therapy reduced the need for blood transfusions by 68%.

4.3 Document D3 describes the use of anti-complement C5 mAb (Eculizumab), corticosteroids, immunosuppressive drugs, and blood transfusions as treatments for the symptoms of PNH.

Documents D1-D3 therefore all anticipate the novelty of the present claims 1-12 and 16-24

4.4 Document D7 discloses the use of anti-human complement C5 (h5G1.1-scFv; Pexelizumab) for the treatment of hemolytic disease (p2504, col.1, li.20-28) and by implication its associated symptoms.

Document D7 thus anticipates the novelty of claims 1,8,12 and 17-24

5.0 Article 56 and 83 EPC

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 6,13-15,18,21 and 25-27 is not sufficiently disclosed according to Article 83 EPC and/or does not involve an inventive step within the meaning of Article 56 EPC.

5.1 Although the description provides data for the use of the anti-C5 antibody

Eculizumab and EPO for treating the effects of hemolytic disease, the use of other molecules grouped in claims 6,14,18,21 and 27 is not disclosed (Article 83 EPC). Given that the other molecules listed lack any similarity to anti-C5 mAb and EPO it cannot be accepted that they also provide solutions for the treatment of the disclosed symptoms. Claims 6,14,18,21 and 27 therefore lack an inventive step (Article 52(1) and 56 EPC).

5.2 The subject matter of present claims 13-15,26 and 27 relates to routine variations that cannot be seen as involving an inventive step according to Article 56 EPC.

5.3 The timing of transfusion independence (present claim 25) cannot be considered to be inventive under Article 56 EPC since there is no evidence in the present application suggesting that the claimed technical effect can be achieved by any other substance to that known in the prior art i.e. anti-C5 mAb.

6.0 Further remarks

The Applicant is informed that a non-unity objection could have been raised in view of the claims as presently formulated since the unifying features: a) linking the different diseases to be treated is considered to be based in that they are all somehow linked to PNH; and b) linking the different alternative molecules which bind/block complement is considered to be based on that they are suitable for treating PNH, are both known in the art (see p2, para.2 of the description, Documents D1-D3).

However, due to the insufficiencies as indicated above under items 3-5 which demand suitable amendments, no objection was held at the search stage, but depending on the amendments made, might become necessary at a later stage.

The examination is being carried out on the **following application documents**

Description, Pages

1-39	as published

Claims, Numbers

1-14	received on	31-01-2011	with letter of	28-01-2011

Drawings, Sheets

1/11-11/11	as published

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia.", HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825, ISSN: 1520-4391

D2 JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough", 19000101, [Online] 13 December 2002 (2002-12-13), pages 1-2, XP009106338, Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle/446336[retrieved on 2008-09-24]

D3 "Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria", TRANSPLANT NEWS, [Online] 15 January 2004 (2004-01-15), XP002497241, Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_m0YUG/is_1_14/ai_n17208612 [retrieved on 2008-09-25]

D4 HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083, ISSN: 0006-4971

D5 THOMAS T C ET AL: "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv", MOLECULAR IMMUNOLOGY, ELMSFORD, NY, US, vol. 33, no. 17-18, 1 January 1996 (1996-01-01), pages 1389-1401, XP002262113, ISSN: 0161-5890

D6 HILLMEN PETER ET AL: "Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria.", THE NEW ENGLAND JOURNAL OF MEDICINE 5 FEB 2004, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406

The following documents are cited by the Examiner. A copy of the documents is annexed to the communication and the numbering will be adhered to in the rest of the procedure.

D9 HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH).", BRITISH JOURNAL OF HAEMATOLOGY, vol. 121, no. Supplement 1, May 2003 (2003-05), page 87, XP009144758, & 43RD ANNUAL SCIENTIFIC MEETING OF THE BRITISH SOCIETY FOR HAEMATOLOGY; GLASGOW, UK; APRIL 07-09, 2003 ISSN: 0007-1048

D10 KAPLAN M: "ECULIZUMAB", CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 1017-1023, XP009045356, ISSN: 1472-4472

1 Introduction

1.1 The Applicant has elected the further examination of invention 4, as set out in our previous communication following an objection under Article 82 EPC.

1.2 The Examining Division considers oral proceedings (pursuant to the request in the Applicants' letter of 28.01.2011) as expedient because the Applicants' arguments are not deemed persuasive.

Oral proceedings are hereby appointed.

Matters to be discussed at oral proceedings

2 Article 123 EPC

The amended claims 1-3,5-10 and 11-14 are not considered allowable under Article 123(2) EPC.

2.1 The only basis provided for the specific dose regimes of claims 1-14 relates to antibody 5G1.1, i.e. eculizumab, and not anti-C5 antibodies in general.

2.2 Claims 1-3, and 8-10 further refer to 'serum complement activity' however, p. 38. li.13 describes only 'cell lysis' being reduced to below 20% of normal serum complement activity.

2.3 Claims 2 and 9 relate to a specific dosage regime, however, no basis can be found that **this specific dosage regime** resulted in the 'cell lysis' being reduced to below 20% of normal serum complement activity. As far as can be ascertained from p.35, li.5-22, only two patients received this dosage schedule. Only in patients receiving 900mg of eculizumab '**every other week**' (p.36, li.18) was 'cell lysis' seen to be reduced to below 20% of normal serum complement activity.

2.4 There is no basis for the general use of an increased dose or increased frequency of dose as stated in claim 3. A basis can only be seen to exist for administering 900mg every 12 days. Furthermore, there is no basis for these features in combination with antibody serum combination and serum complement activity.

2.5 Claim 10 referring to an amount effective to maintain a serum concentration of antibody around 35 µg/ml has no basis in the description. Basis only exists for the administration of **900mg eculizumab every 12 days** to maintain levels **above** 35µg/ml.

3 Articles 83 and 84 EPC

The application does not meet the requirements of Articles 83 and/or 84 EPC, because claims 1-3 and 8-10 are not clear, and/or lack support and disclosure.

3.1 Rule 43(2) EPC states that a European patent application may only contain more than one independent claim in the same category if the subject matter of the application relates to either: a plurality of interrelated products; different uses of a product; or alternative solutions to a particular problem, where its is inappropriate to cover these alternatives by a single claim. In the present case, however, claims 1-3 and 8-10 all embody related to solutions to the same problem, i.e. the provision of a dosage schedule for PNH.

3.2 The application provides experimental details of dosage optimisation of an anti-C5 antibody, 5G1.1 eculizumab, for the treatment of PNH in a group of 11 patients. Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention. A lack of support and disclosure therefore occurs for present claims 1-3 and 8-10 over their broad scope.

3.3 Claims 3 and 10 do not meet the requirements of Article 84 EPC in that the matter for which protection is sought is not defined. The claim attempts to define the subject-matter in terms of the result to be achieved. Such a definition is only allowable under the conditions elaborated in the Guidelines C-III, 4.10. In this instance, however, such a formulation is not allowable because it appears possible to define the subject-matter in more concrete terms, viz. in terms of how the effect is to be achieved, i.e. the administration schedule required.

3.4 The relative term 'about' used in claim 10 has no well-recognized meaning and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 84 EPC).

4 Priority

The priority document does not appear to disclose the subject matter of the present claims 3 and 10, relating to the dosing schedules for the maintenance of a serum concentration of antibody above 35 µg/ml. The P document D6 is therefore citable as prior art.

5 **Article 54 EPC**

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 3 and 10 is not new within the meaning of Article 54(1) and (2) EPC.

5.1 D6 discloses the use of a 900mg dose of eculizumab every 12 days for the maintenanceof a serum concentration of 35 µg/ml (p.555, col.1, §4 - p.556, col.1, §1).

D6 thus anticipates the subject-matter of present claims 3 and 10.

6 **Article 56 EPC**

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1-14 does not involve an inventive step within the meaning of Article 56 EPC.

6.1 Document D2 is considered to be the closest prior art to the subject-matter of claim 1 and discloses a trail for the treatment of PNH, comprising administering 600mg eculizumab/patient/week for four weeks, followed by 900mg/patient every two weeks.

The subject-matter of claim 1 therefore differs from this known D2 in that the same dose is administered every 12 days.

The technical effect resulting from this difference would appear to be the prevention of relapse in 10% of the patients where hemolytic activity returned.

The problem to be solved by the present invention may therefore be regarded as the optimisation of a known dosing schedule for individual patients.

The solution proposed in claim 1 of the present application cannot be considered to involve an inventive step (Articles 52(1) and 56 EPC), for the following reasons:

The use of eculizumab for the treatment of PNH is well known in the prior art (see D1-D4 and D9) as well as useful dosage regimes (D2 and D9). It would therefore be obvious to the skilled person with a knowledge of present dosage regimens for PNH described in D2 and D9, and presented with a patient

displaying relapse, to adjust the dosage regimen through increased dose or frequency. In particular, it was already known from D10 that a single dose of eculizumab could block complement activity for **up to** two weeks (p.1019, col. 2, §2), therefore 14 days would be considered an extended interval that the skilled person would reduce upon observing relapse.

The dose regimen claimed is therefore considered to constitute a routine improvement with no new surprising effect.

6.2 For the same reasons as stated above (see, 6.1), claims 2,3 and 8-10 can also not be considered inventive.

6.3 Dependent claims 4-7 and 11-14 do not appear to contain any additional features which, in combination with the features of any claim to which it refers, meet the requirements of the EPC with respect to novelty and/or inventive step.

7 Concluding Remarks

7.1 The Applicant may file new claims which take in to account the above comments, however, on the basis of the present application documents, refusal of the application under Article 97(2) is to be expected. The Applicants' attention is therefore drawn to the possibility of requesting an appealable decision according to the state of the file (Guidelines E-X, 4.4).

7.2 In order to facilitate the examination of the conformity of any amendments to the application with the requirements of Article 123(2) EPC, the Applicant is requested to clearly identify such amendments, irrespective of whether they concern amendments by addition, replacement, or deletion, and to indicate the passages of the application as filed on which these amendments are based.

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a **printed** copy of the relevant parts of the application as filed.

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23. Sep. 2011

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Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

München, September 23, 2011
WA/ARO/FLG

This is in reply to the Summons to attend oral proceeding issued in the above-mentioned case. The indication of the chairman to accept written submission until September 23 2011 is greatly appreciated.

Enclosed is a main request, a new first auxiliary request, a new second auxiliary request, a new third auxiliary request and a new fourth auxiliary request. These requests replace the previously filed claim set.

1. AMENDMENTS TO THE CLAIMS (Rule 137(4) EPC)

1.1. Main Request and Auxiliary Request I to III

Support for the amendments to the claims constituting the first auxiliary request, the second auxiliary request, and third auxiliary request is indicated in the following Table.

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New Claim #	Previous claims and passages in the description supporting the Main Request	Previous claims and passages in the description supporting the Auxiliary Request 1	Previous claims and passages in the description supporting the Auxiliary Request 2	Previous claims and passages in the description supporting the Auxiliary Request 3
1	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1 and page 38, lines 11-17	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and page 37, line 10 to page 38, line 16	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claim 4	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claims 1 and 4
2	1	1	1	2
3	2	2	2	3
4	3	3	3	5
5	page 37, line 10 to page 38, line 16	5	page 37, line 10 to page 38, line 16	6
6	5	6	5	7
7	6	7	6	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claims 8 and 11
8	7	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and page 37, line 10 to page 38, line 16	7	9
New Claim #	Corresponding previous claims/ support for Main	Corresponding previous claims/ support for Auxiliary 1	Corresponding previous claims/ support for Auxiliary 2	Corresponding previous claims/ support for Auxiliary 3

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9	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1 and page 38, lines 11-17	8	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claim 11	3
10	8	9	8	12
11	9	3	9	13
12	3	12	3	14
13	page 37, line 10 to page 38, line 16	13	page 37, line 10 to page 38, line 16	
14	12	14	12	
15	13		13	
16	14		14	

1.2. Auxiliary Request IV

1.2.1. New claim 1 is based on claim 1 as previously filed. The term " to thereby maintain in the patient a serum complement activity at a level below 20% of the normal serum complement activity" has been deleted. The anti-C5 antibody has been further defined as being eculizumab (i.e. also designated as antibody h5G1.1 in the application). The deletion of said term and the use of eculizumab are supported at page 4, line 17 to page 5, line 5 of the application as filed.

The term "at least" has been deleted and the administration has been further defined as intravenous administration. Support for the dosage of eculizumab recited in new claim 1 has been indicated in section 1.2. of our submission of January 28, 2011. Further support can be found at page 35, lines 5-22 of the application as filed.

1.2.2. New claims 2 to 4 correspond to claims 5 to 7 as previously filed.

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1.2.3. New claims 5 to 8 are based on new claims 1 to 4 and have been rephrased in the "Swiss-type" claim format. In accordance with decision G 1/09 the applicant is allowed to also use the "Swiss-type" claim format in the present application.

2. INADMISSIBLE BROADENING (Article 123(2) EPC)

2.1. Antibody 5G1.1

2.1.1. *Main Request*

At page 3 of the Office Action, claims 1-3 and 5-14 were objected to as allegedly lacking written description. According to the Examining Division, "the only basis provided for the specific dose regimes of claims 1-14 relates to antibody 5G1.1, i.e. eculizumab, and not anti-C5 antibodies in general." Applicant disagrees.

The instant claims, as amended, are drawn to novel, inventive, and fully-supported uses of an anti-C5 antibody to prevent serum complement activity breakthrough in a patient afflicted with PNH. *See* specification at, e.g., page 5, lines 6 to 10. *See also* specification at page 4, line 20 to page 5, line 1 (describing use of anti-C5 antibodies such as eculizumab and pexelizumab). For example, claim 1 of the Main Request is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity.

As acknowledged by the Examining Division, the specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal. These particular approaches are embraced by claims 2, 3, 10, and 11, as well as the claims dependent therefrom; accordingly, these claims are believed in view of the remarks of the Examining Division to *per se* satisfy the requirements of Article 123 EPC.

Yet, aided by the express teachings of the specification, a skilled artisan at the effective filing date of the application would have appreciated that there are a number of therapeutic anti-C5 antibodies and associated therapeutic approaches

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that could be used to prevent serum complement activity breakthrough in PNH patients as claimed. In fact the specification describes pexelizumab in addition to eculizumab. With the benefit of the teachings of the specification, the skilled artisan would have understood that other therapeutic regimens (dosing amounts and intervals) were available for the claimed use with pexelizumab – as well as other anti-C5 antibodies – to maintain serum complement activity of PNH patients at a level below 20% of normal complement activity.

In view of the foregoing, it is clear that the skilled artisan would have at once unambiguously appreciated that Applicant provided in the as-filed specification a clear basis for the entire scope of the subject matter claimed. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

2.1.2. *Auxiliary Requests II to IV*

Auxiliary Requests II to IV recite "eculizumab" in all of the claims, thus, rendering the rejection moot.

2.2. <u>The term "a serum complement activity level below 20% of normal serum complement activity"</u>

2.2.1. Claims 1-3 and 8-10 were also objected to as allegedly being drawn to new matter. Specifically, the Examining Division urges that the specification does not provide descriptive support for "serum complement activity...being reduced to below 20% of normal serum complement activity." Applicant disagrees.

2.2.2. The instant claims involve the use of an anti-C5 antibody to maintain in PNH patient serum a complement activity at a level below 20% of the normal serum complement activity. Applicant respectfully points out the express support for this claim language at, e.g., page 38, lines 11-17 and Fig. 8 of the specification, in which the inventors describe the physiological effects of eculizumab treatment of PNH over time. Specifically, the specification states that "serum hemolytic activity (i.e. ...cell lysis)...was dramatically reduced in the majority of patients to below 20% of normal serum complement activity under eculizumab treatment." *Id.* Thus, as an initial matter, this passage makes clear that the inventors use the terms "serum complement activity", "cell lysis", and "serum hemolytic activity"

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interchangeably. The interchangeability of these terms becomes even more clear when viewed in the context of the experiments set forth in the application.

For example, at pages 37 and 38 of the specification, the inventors describe the results of experiments in which patient serum was tested for its complement activity as a function of its ability to lyse red blood cells (hemolysis) *in vitro*. This test, known as a hemolysis assay, involves incubating chicken erythrocytes with serum from healthy individuals (normal serum) or serum from eculizumab-treated PNH patients (patient serum). In the presence of normal serum that contains a full battery of complement component proteins, the chicken erythrocytes are lysed thereby releasing hemoglobin from the cells (as determined by optical density). By contrast, patient serum, in which eculizumab has, in effect, inhibited the activity of complement component C5, hemolysis is reduced and thus less hemoglobin is released. Following this assay, the inventors determined the percent hemolysis in patient serum as compared to normal serum essentially by dividing the amount of hemoglobin released from erythrocytes in patient serum (patient serum complement activity) by the amount of hemoglobin released from erythrocytes in normal serum (normal serum complement activity), multiplied by 100. The skilled artisan aware of hemolysis assays would have readily understood that "percent hemolysis" is just another way of expressing "percent cell lysis" or "percent serum complement activity" in patients as compared to corresponding normal human serum, the latter of which being recited in the claims of the Main Request.

2.2.3. In T 0878/09, the Board recently made clear that the Examining Division's

> "determination of whether or not the provisions of Article 123(2) EPC are fulfilled requires the determination of 'subject matter'." The Board further cautioned that "[s]ince the same subject-matter may be described by different words – for example the same glass may be described as either 'half full' or 'half empty' – a difference in wording does not per se necessarily establish that the subject-matter defined by the words is different."

In this case, the Examining Division objects to a linguistic distinction without a difference in meaning. The skilled artisan reading the instant application would have at once clearly and unambiguously appreciated that serum "cell lysis" and "serum complement activity", as used by the inventors, are equivalent terms. Thus, Applicant submits that the objected to claim language was fully defined

and described in the specification as filed such that a skilled artisan would have readily understood the metes and bounds of the claims. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

2.3. Dose as stated in claim 3

2.3.1. The Examining Division alleges that" [t]here is no basis for the general use of an increased dose or an increased frequency of dose as stated in claim 3."

2.3.2. Applicant respectfully disagrees. However solely in the interest of expediting prosecution of this application, the amendments to claim 3 (now claim 4) are believed to render the objection moot.

2.4. A serum concentration of antibody around 35 µg/ml

2.4.1. The Examining Division alleges that "[c]laim 10 referring to an amount effective to maintain a serum concentration of antibody around 35 µg/ml has no basis in the description."

2.4.2. Applicant respectfully disagrees. However solely in the interest of expediting prosecution of this application, the amendments to claim 10 (now claim 12) are believed to render the objection to claim 10 moot.

2.5. Auxiliary Request IV

The subject-matter of the claims of the fourth auxiliary request closely adheres to and reflects the results of the application as filed, in particular at page 32, lines 12-16 and page 35, lines 5-22 of the application as filed. Moreover, this request is confined to the dosage considered supported by the Examining Division.

3. SUFFICIENCY OF DISCLOSURE, CLARITY, CONCISENESS AND SUPPORT (Articles 83 and 84 EPC)

3.1. Independent claims of the same claim category (Rule 43(2) EPC)

3.1.1. At page 4 of the Office Action, the Examiner invoked Rule 43(2) EPC to object to the claims as having more than one independent claim in the same category.

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3.1.2. While Applicant does not concede to any aspect of the stated reasons for objection, the instant claims contain only two independent claims (claims 1 and 9), each of which is drawn to a different category, i.e., claim 1 (composition-for-use) and claim 9 (Swiss-type use). In accordance with decision G1/09 the applicant is allowed to use the format of a second medical use claim under the EPC 2000 and the "Swiss-type" claim format in the present application. The corresponding new claims 1 and 9 are the only independent claims.

In view of the instant amendments, the objection to the claims is rendered moot.

3.2. The antibody h5G1.1

3.2.1. *Main Request*

In this aspect of the Office Action, the Examiner again objects to the independent claims because they are not limited to eculizumab. According to the Examining Division, "[g]iven the different affinities, functions and pharmacokinetics of individual specific antibodies, the establishment of an optimal dosing schedule will be dependent on the antibody used." At bottom, the Examiner contends that, in contravention of Rules 43(1) and 43(3) EPC, the independent claims allegedly do not "contain all the technical features essential to the definition of the invention." Office Action at page 4. Applicant respectfully disagrees.

Rule 43(1) requires that the claims define the subject matter claimed in terms of "technical features which are necessary for the definition". Rule 43(3) further specifies that a broad claim setting forth the necessary technical features may be followed by additional claims drawn to particular embodiments of the broader claim. The instant claims amply satisfy these requirements.

As noted above, claim 1 is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity. This independent claim, as with its sister claim 8 in the Swiss-type use format, is defined by, among other things, structure, e.g., an anti-C5 antibody, and affirmative step – that the antibody is to be administered to a patient to maintain a

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serum complement activity level below 20% of the normal serum complement activity. The claim further defines the particular patient population – PNH patients – to which the uses are directed and particular pathological state – serum complement activity breakthrough – sought to be averted.

Under the guidance of these numerous essential features, a skilled artisan at the effective filing date of the application would have recognized, as did the inventors, that there are a number of therapeutic anti-C5 antibodies (e.g., eculizumab and pexelizumab) and associated therapeutic approaches that could be used to prevent serum complement activity breakthrough in PNH patients as claimed. The specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal.

In accordance with Rule 43(3), claims 2, 3, 10, and 11 of the Main Request further define particular embodiments of the broader claim, particularly the use of eculizumab under particular dosing regimens. The Examiner has provided no reason as to why a skilled artisan, in view of the essential technical features set forth in the claims, would not have readily appreciated that Applicant was in full possession of the claimed uses or would not have been able to practice the claimed uses using nothing more than the teachings of the specification, the knowledge in the art, and routine experimentation. Thus, Applicant respectfully submits that the instant claims amply satisfy the requirements of Article 84 and Rules 43(1) and 43(2), in contrast to the position advanced by the Examining Division. Reconsideration and withdrawal of the objection are respectfully requested.

3.2.2. *Auxiliary Requests II to VI*

Auxiliary Requests II to VI recite "eculizumab" in all of the claims, thus, rendering the rejection moot.

3.3. Result-to-be achieved

3.3.1. At page 4 of the Office Action, claims 3 and 10 were objected to as allegedly lacking written description. According to the Examiner, claims 3 and 10

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impermissibly attempt to define subject matter in terms of a result to be achieved allegedly in contravention of Article 84 EPC and the Guidelines for Substantive Examination, specifically Chapter III, 4.10 [hereinafter the Guidelines].

3.3.2. Applicant respectfully disagrees with the Examiner's characterization of the claims. However, solely in the interest of expediting prosecution, the present amendments to claims 3 and 10 are believed to render the objections moot.

In the event that the comments in the Summons have bearing on any of the instant claims, Applicant provides the following additional remarks.

According to the Guidelines, claims defined by results to be achieved are allowable if: (a) the invention can only be defined in such terms or cannot otherwise be defined more precisely without unduly restricting the scope of the claims and (b) the result is one which can be directly and positively verified by tests or procedures adequately specified in the description or known to the person skilled in the art and which do not require undue experimentation. Even assuming *arguendo* that the instant claims are defined by a result to be achieved, Applicant respectfully submits that the instant claims nonetheless amply satisfy these legal requirements for allowability.

With respect to the first prong of the aforementioned test, Applicant again reiterates that the instantly amended claims are drawn to novel, inventive, and fully-supported uses of an anti-C5 antibody to prevent serum complement activity breakthrough in a patient afflicted with PNH. Claim 1, for example, is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity.

As acknowledged by the Examining Division, the specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal. The Examiner insists that the claims should go no further. Yet, in view of the teachings of the specification, a skilled artisan at the effective filing date of the application would have appreciated that eculizumab is not the only anti-C5 antibody that is useful to

prevent serum complement activity breakthrough in PNH patients as claimed. Indeed the specification provides another example of an anti-C5 antibody, pexelizumab, which is useful in accordance with the claims.

Thus, the contribution of the disclosure and indeed the scope of the claimed invention is much broader than a single antibody and, accordingly, would be unduly limited if Applicant was required, as urged by the Examining Division, to limit the claims to eculizumab. As noted above, the instant claims are not without definition. In fact, the claims set forth, among other things, the structure of the therapeutic agent used, the affirmative step required for the use, the patient population to which the use is directed, the particular pathological state sought to be avoided, and a measurable, concrete physiological condition to be reached in the patients. Thus, the skilled artisan would have appreciated that like an ashtray for automatically extinguishing a cigarette based on the shape and relative dimensions of the ashtray, the instantly claimed uses "vary considerably in a manner difficult to define whilst still providing the desired effect." The Guidelines, Chapter III, 4.10. Yet the teachings of the specification and the essential features set forth in the claims provide "adequate directions to enable the reader to determine the required dimensions by routine test procedures" and demonstrate that Applicant had possession of the claimed invention at the effective filing date. *Id.*

Moreover, the specification provides and actually exemplifies methods for directly and positively determining whether serum complement activity has reached or is maintained at a level below 20% of the normal serum complement activity. For example, at page 37, line 10 to page 38, line 16 and Figure 8, the inventors disclose the experimental procedures and results of those procedures evaluating the percentage of serum complement activity in PNH patients over time. In view of this, it is abundantly clear that a skilled artisan could have readily and easily practiced the claimed uses using no more than the teachings of the specification, the knowledge in the art, and routine experimentation.

In view of the foregoing, Applicant respectfully submits that the instant claims amply satisfy the requirements of Article 84 EPC and the Guidelines cited by the Examiner. Accordingly, reconsideration and withdrawal of the objection are respectfully requested.

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3.3.3. *Auxiliary request IV*

The claims of fourth auxiliary request do not make use of features characterized by the Examining Division as "result-to-be-achieved".

3.4. The term "about"

3.4.1. At page 4, the Examining Division objected to the word "about" used in claim 10.

3.4.2. The amendments to claim 10 (now claim 12) are believed to render this objection moot.

4. PRIORITY AND NOVELTY (Articles 87 and 54 EPC)

4.1. At page 5 of the Office Action, the Examiner objected to claims 3 and 10 as allegedly being anticipated by D6. According to the Examining Division, the priority document allegedly does not disclose "dosing schedules for the maintenance of a serum concentration of antibody above 35 µg/mL...[and thus] D6 is...citable as prior art." While Applicant does not concede to any aspect of the Examiner's stated reasons for objection, claims 3 and 10 have been cancelled thereby rendering the objection moot.

4.2. The instant claims find full written description support in the priority document [see, e.g., U.S. patent application serial no. 10/771,552 at page 4, lines 16-21; page 5, lines 3-7; and page 34, lines 13-19] and thus D6 is not available as prior art with respect to these claims.

4.3. The claims of the fourth auxiliary request do not refer to the feature "effective to maintain a serum concentration of the antibody of above 35 µg/ml". Applicant thus submits that the new claims are entitled to the claimed priority and consequently D6 is not available as prior art with respect to the new claims.

5. INVENTIVE STEP (Article 56 EPC)

5.1. At pages 5 and 6 of the Office Action, claims 1 to 14 were objected to as allegedly lacking inventive step over D2 and D9, in view of D1, D3, and D4.

According to the Examining Division, the instant claims represent nothing more than the "optimisation of a known dosing schedule for individual patients". Applicant disagrees with this characterization of the claimed invention.

5.2. As noted above, the instant claims are drawn to a novel and inventive use of an anti-C5 antibody to prevent serum complement activity breakthrough in PNH patients. The claims are based, at least in part, on the discovery by the inventors that the devastating hemolytic paroxysms experienced by PNH patients can be effectively controlled by maintaining the patients' serum complement activity at a level below 20% of normal serum complement activity. Failure to maintain serum complement activity below 20% resulted in breakthrough and resumption of the associated debilitating and life-threatening sequelae associated with PNH. As stated in the description of the instant application, this "[b]reakthrough of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with a return of serum hemolytic activity." *See* specification at page 35, lines 8 to 22.

In view of these observations, the inventors appreciated that administering a complement inhibitor (e.g., an anti-C5 antibody such as eculizumab) to a PNH patient in a manner that consistently maintains this experimentally determined low level of serum complement activity is necessary to consistently prevent breakthrough in patients afflicted with PNH. Claim 5 of the Main Request, further requires testing, subsequent to administration of the antibody, to confirm that the reduced serum complement activity has been maintained in the PNH patients. Thus, one of skill in the art at the effective filing date of the application would have immediately recognized from the specification that the claimed invention does not merely represent routine dosage optimization; rather, the claimed invention captures a new therapeutic modality and approach to PNH based on the recognition of the precise physiological levels of complement activity required to stave off PNH-related pathologies. When read through this lens, the cited references, alone or in combination, clearly fail to deprive the instant claims of inventiveness. Applicant describes each of the cited references below.

D2 is a short article related to the preliminary results of a clinical trial in which eculizumab was administered to patients with PNH. D9 is a scientific meeting abstract that, like D2, discloses some preliminary results of a clinical trial related

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to eculizumab. The authors of D9 note, for example, that eculizumab was useful in reducing the need for transfusion in patients with PNH. These references, however, are silent on the need to maintain PNH patient serum complement activity at a level below 20% of normal complement activity or testing for such activity. There is certainly no disclosure or even a suggestion of breakthrough in D2 or D9, that maintaining serum complement activity below 20% of normal could prevent breakthrough, or even of the specific applications (e.g., the specific dosage regimens) of the instantly claimed invention embodied in claims 2 and 3 of the Main Request.

D3 is a short article related to approval of orphan drug status for eculizumab in PNH treatment. D4 is a scientific manuscript relating to the use of warfarin to inhibit thrombosis in PNH patients. These references, even more so than D2 and D9, fail to disclose or suggest any specific treatment conditions for an anti-C5 antibody, let alone the need to maintain PNH patient serum complement activity at a level below 20% of normal complement activity or testing for such activity. There is certainly no disclosure or even a suggestion in D3 or D4 of breakthrough or that maintaining serum complement activity below 20% of normal could prevent breakthrough as claimed.

D10 is a review article summarizing the generation and characteristics of eculizumab as a therapeutic agent. D10 discloses that eculizumab may be therapeutically beneficial in conditions such as, but not limited to, myocardial infarction, ischemia-reperfusion injury, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). *See* D10 at, e.g., page 1019. The reference further notes that Alexion (the instant Applicant) was at the time of the reference's publication engaged in clinical trials evaluating the use of eculizumab in, e.g., RA, SLE, psoriasis, and lupus nephritis. The Examiner cites to D10 for the purpose of establishing the activity of a single dose of eculizumab in patients with SLE. However, D10 does not disclose or suggest the use of an anti-C5 antibody to prevent serum complement activity breakthrough in patients afflicted with PNH. In fact, D10 does not relate to PNH at all. There is certainly no recognition by the authors of D10 that maintaining a level of serum complement activity below 20% of normal is necessary to consistently prevent breakthrough in patients afflicted with PNH.

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D1 published in December 2004 as reflected on the face page of the journal issue in which the article appeared (attached). As a December 2004 publication date is well after the priority date of the instant application (February 3, 2004), D1 is simply unavailable as prior art against the instant claims.

Thus, none of the cited references disclose or suggest maintaining PNH patient serum complement activity at a level below 20% of normal complement activity.

In the event that the foregoing arguments are not accepted by the Examining Division, Applicant submits the following arguments relating to specific dependent claims and the Auxiliary Requests I to III for consideration. Claims 2, 3, 10 and 11 (and all claims of Auxiliary Request 3) recite that eculizumab is to be administered to the patient at least once every 12 days at a dose of at least 900 mg. The fact that the PNH symptoms of all of the treated patients in the study of the Applicant were prevented by using this dosing regimen was surprising. The skilled artisan would not have foreseen this result based on any combination of the cited art. Nor would the skilled artisan have had any reason to modify the dosage regimen of 900mg of eculizumab every 14 days that was recited in D2.

Furthermore, claims 5 and 13 (and all claims of Auxiliary Request 1) recite that the patient's serum complement activity is to be tested to determine if the serum complement activity is maintained at a level below 20% of normal serum complement activity. Testing the patient's serum complement activity is not recited in any of the art cited by the Examining Division. At the priority date, one of ordinary skill in the art would not have been motivated to test the patient's serum complement activity because there was no recognition in the art that maintaining a level of serum complement activity below 20% of normal is necessary to consistently prevent breakthrough in patients afflicted with PNH.

In view of all of the foregoing arguments, Applicant respectfully submits that the instant claims represent a therapeutically useful, novel, and inventive approach over the cited prior art. Accordingly, reconsideration and withdrawal of the objection are respectfully requested.

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5.3. *Auxiliary request IV*

The arguments presented above apply *mutatis mutandis* to the fourth auxiliary request.

It is moreover emphasized with regard to the fourth auxiliary request that the issue in assessing inventive step is not whether the skilled person could have arrived at the invention by adapting or modifying the closest prior art, but whether he would have done so because the prior art incited him to do so in the hope of solving the objective technical problem or in expectation of some improvement or advantage.

D2 fails to disclose the administration of 900 mg eculizumab once every 12 days. It is concluded in D2 that the used dosage regimen of 900 mg eculizumab once every 14 days is effective in the treatment of PNH.

As regards D10, we note that this disclosure does not all refer to a specific dosage regimen of eculizumab in the treatment of PNH. It is emphasized that D10 refers to the treatment of SLE (systemic lupus erythematosus). The dose used for the treatment of SLE cannot provide any information about the treatment of PNH. Moreover, a document referring to a different medical indication does not qualify as secondary prior art. If it were nevertheless to be considered, we submit that the skilled person does not find any suggestion therein for a fine-tuning of dosage in the treatment of PNH.

Also the remainder of the cited prior art does not provide any incentive to administer 900 mg eculizumab every 12 days.

The inventors have thus surprisingly found that - upon the initial dosage regimen step (i) and (ii) – as step (iii) 900 mg eculizumab every 12 days is required in order to effectively and consistently block serum hemolytic activity; cf. page 35, lines 5-22. In more detail, 900 mg eculizumab every 14 days is not sufficient to block complement break-through in all patients. The fact that 900 mg eculizumab every 14 days may suffice in most patients to block complement break-through cannot be detrimental to inventive step, because only the dosage regimen according to the invention ensures that all patients are successfully treated.

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This dosage regimen is technically advantage as compared to the use of 900 mg eculizumab every 14 days (D2). As disclosed at page 35, lines 5-22 of the application as filed 900 mg eculizumab every 14 days are not sufficient in order to effectively and consistently block serum hemolytic activity in PNH patients.

Taken together only the result of presented in the examples of the present application would led the skilled person to a dosage of 900 mg eculizumab every 12 days. It is therefore submitted that the claims of the fourth auxiliary request are inventive.

6. FURTHER REQUESTS

With the above amendments and explanations, it is submitted that the new main request meets the requirements as set forth in the Summons. In case the Examining Division should come to the conclusion that the main request is not patentable, consideration of the first auxiliary request, the second auxiliary request, third auxiliary request and the fourth auxiliary request (in that order) is requested.

If the Examining Division shares this view, then it would seem that it were no longer necessary to hold oral proceedings. In this case it is requested that oral proceedings be cancelled and the proceedings be continued in writing. Otherwise, our request for oral proceedings according to Article 116(1) EPC is maintained. In the latter case it is requested that filing of at least one further request during oral proceedings is allowed. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the telephone.

Dr. Roger Abseher
European Patent Attorney

Enclosure:
New claim sets

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16. Nov. 2012

EP 11 00 1632.6 - 2123
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/1

München, November 15, 2012
WA/ROA/BUK

This is in reply to the Communication pursuant to Art. 94(3) EPC dated July 10, 2012:

1 AMENDMENTS TO THE CLAIMS

1.1. New claims 1 to 3 correspond to previous claims 1 to 3.

1.2. New claim 4 is based on previous claim 4 and has been made into a dependent claim referring back to claims 1 to 3. Said reformulation is supported by previous claim 4.

1.3. New claim 5 corresponds to previous claim 5.

1.4. New claim 6 is based on previous claim 6. The claim now only refers to an anti-C5 antibody.

1.5. New claims 7 to 19 correspond to previous claims 7 to 19.

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1.6. New claim 20 is based on previous claim 20 and now properly refers back to the subject-matter of claims 1 to 3.

1.7. New claims 21 to 23 correspond to previous claims 21 to 23.

2 INADMISSIBLE BROADENING (ARTICLE 123(2) EPC)

2.1. Claims 1 to 3

2.1.1. In sections 1.1 of the Communication, the Examining Division objects to the feature that the patient has been vaccinated against Neisseria meningitides or the claimed pharmaceutical composition is administered to patients in conjunction with a vaccine against Neisseria meningitides in claims 1 to 3. According to the Examining Division, support for these claims is derived from the working examples and "a feature taken from an example cannot be generalised to the general teachings of the invention without infringing Article 123(2) EPC."

2.1.2. Applicant cannot subscribe to the assessment of the Examining Division and submits the following. The new claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The anti-C5 antibody inhibits cleavage of complement component C5 into fragments C5a and C5b. The claims also require that the patients be vaccinated against *Neisseria meningitides* (see claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (see claims 2 and 3).

2.1.3. The compositions as recited in the claims are supported at, e.g., page 5, lines 3 to 9 of the specification (WO 2007/106585). The use of such compositions in the treatment of patients with hemolytic diseases is disclosed at, e.g., page 8, lines 19-21 and page 22, lines 7-17. Thus, the question is whether the specification provides support for the introduction of the feature of vaccination

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against *Neisseria meningitides* as mentioned in the example section which is alleged to constitute an undue generalization. As indicated in detail below, Applicant submits that the specification clearly supports the presently claimed subject matter.

2.1.4. With regard to the objective of Article 123(2) EPC and the question of allowable generalization, a considerable body of case law has developed and has partially been manifested in the Guidelines for Examination in the European Patent Office (hereinafter referred to as "Guidelines") to ensure that the claims of a European patent application are not amended in such a way that they contain subject-matter which extends beyond the content of the application as filed.
In relation to the compliance of a correction under Rule 43(2) EPC to Art. 123(2) EPC it was ruled that if it

> "expresses what a skilled person, using common general knowledge, would already derive on the date of filing from the parts of a European patent application, seen as a whole" (G11/91, reasons for the decision, paragraph 4)

a correction would not infringe Art. 123(2) EPC.

According to the Guidelines at Part H, Chapter IV, 2.2, an amendment fails to comply with Article 123(2) EPC if it

> "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." (Guidelines, Part H, Chapter IV, 2.2)

The Guidelines further clarify that implicit subject matter is

> "no more than the clear and unambiguous consequence of what is explicitly mentioned in the application as filed." (Guidelines, Part H, Chapter IV, 2.3)

2.1.5. As evident from the above, compliance with Article 123(2) EPC does not require *ipsis verbis* support in the description but extends to subject-matter being

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explicitly disclosed in other terms or implicitly disclosed. For example, in T 0878/09 (reasons for the decision, paragraph 29) the Board stated that the

> "determination of whether or not the provisions of Article 123(2) EPC are fulfilled requires the determination of 'subject matter'....[and s]ince the same subject-matter may be described by different words – for example the same glass may be described as either 'half full' or 'half empty' – a difference in wording does not per se necessarily establish that the subject-matter defined by the words is different."

2.1.6. It is further not categorically the case that a specific feature taken from the specification cannot be generalized to a more general invention without infringing Article 123(2) EPC. Rather, the Board has held that Article 123(2) is not satisfied in cases where, for example, the as-filed specification: (i) describes a specific embodiment of a claimed invention and (ii) the general applicability of that embodiment would not have been evident to a person of skill in the art reading the specification (see T157/90). In the latter decision, the claims related to a calcitonin polypeptide comprising an additional amino acid at the C-terminus. The subject application taught that the additional amino acid (+1 amino acid) was required for the successful amidation of a proline residue preceding the additional amino acid. Amidation of the proline was "a feature required for biological activity" of the calcitonin polypeptide. However, the detailed description of the invention described only one embodiment: that the glycine residue (+1) is in vivo required during processing events for the amidation of the adjacent carboxyl terminal amino acids. The Board concluded that the specification, even when viewed in the context of the knowledge in the art at the effective filing date of the application, taught, if anything, that glycine is the amino acid necessary for the enzymatically processing of the precursor to arrive at the necessary amidation of the proline (see Reasons for Decision, paragraph 2.11). In fact, there was evidence of record that even glycine was not actually required for processing of calcitonin. Thus, according to the Board, disclosure of the sole embodiment glycine, without more, would not have been enough for a skilled artisan to recognize that any amino acid could be used as an amidation signal in the processing of calcitonin.

2.1.7. In a further decision T17/86 (confirmed by T284/94 and T470/05) concerning the question whether a technical feature claimed separately from the disclosed

combination with others may be introduced into a claim without contravening Art. 123(2) EPC it was ruled that this is allowable, if it is

> "evident beyond any doubt to a skilled person reading the original description that this isolated feature on its own enables the object in view to be achieved." (T1786, reasons for the decision, paragraph 3)

and with reference to a claim for a device that it should

> "be ascertained whether the application as filed indisputably disclosed all the technical features of the device covered by the amended claim and whether it is clear beyond any doubt to a skilled person that the device can function independently of any other added element to achieve the object of the invention as now claimed." (T1786, reasons for the decision, paragraph 3)

2.1.8. The existing case law regarding this issue has been integrated into the Guidelines in the form of three requirements that need to be met (see Part H, Chapter V, 3.2.1):

a) the feature is not related or inextricably linked to the other features of that embodiment, and

b) the removal of the omitted features from the embodiment passes the essentiality test:

   i. the feature was not explained as essential in the disclosure;

   ii. the feature is not, as such, indispensible for the function of the invention in the light of the technical problem the invention serves to solve; and

   iii. the replacement or removal requires no real modification of other features to compensate for the change,

   and

c) the overall disclosure justifies the generalizing isolation of the feature and its introduction into the claim.

2.1.9. In decision T190/99, the Board has also stated that

> "the skilled person when considering a claim should rule out interpretations which are illogical or which do not make technical sense. He should try, with synthetical propensity i.e. building up rather than tearing down, to arrive at an interpretation of the claim which is technically sensible and takes into account the whole disclosure of the

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> patent. The patent must be construed by a mind willing to understand not a mind desirous of misunderstanding." (T 0190/99, Reasons for the Decisio, paragraph 2.4).

2.1.10. Out of the foregoing law, several intertwined principles emerge. First, neither the Guidelines nor the case law articulates an Article 123(2) EPC analysis that is inflexible or narrow; instead, the analysis considers the context in which the amended claims sit, i.e., the teachings of the patent application disclosure as a whole and what those teachings mean implicitly within the milieu of the common scientific knowledge in the art. Second, the analysis must be conducted from the viewpoint of a skilled person who is interested in, and "willing to understand", the disclosure and what it teaches him/her.

2.1.11. Under this analysis, it becomes evident that a skilled person reading the instant disclosure would have immediately recognized that the specification clearly defined not only a population of patients vaccinated against meningitis, but therapeutic compositions used in conjunction with such vaccination.

2.1.12. Specifically, both the original claims and the specification itself provide a supportive context for the instant claims. The skilled person would have readily appreciated that the primary aim of the specification is providing therapeutic solutions to the problem of hemolytic diseases such as paroxysmal nocturnal hemoglobinuria (PNH). For example, in its *Background* section starting at page 1, the application begins with a detailed discussion of the problem of PNH. The skilled person was clearly aware, as were the inventors of the invention covered by the pending application, of the need in the art at the filing date of the application for "therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH" (see *Background* section). Likewise, the skilled person would have immediately recognized that the numerous treatment modalities set forth in, and exemplified by, the application – particularly using compositions comprising an antagonist anti-C5 antibody – are an answer to this need.

2.1.13. Not only does the specification provide this array of therapeutic approaches, but the skilled artisan would have also appreciated that a number of specific patient populations are identified by the inventors as being amenable to such

approaches. These patient populations include, e.g., patients having hemoglobin levels less than 14 g/dL if a man, or less than 12 g/dL if a woman (see specification at, e.g., page 3, lines 12 to 14). Other patient populations described are, e.g., those who are anemic or those who remain anemic after treatment with a composition comprising an antagonist anti-C5 antibody. (see specification at, e.g., page 3, lines 3 to 13). Indeed, the general class of patients with PNH is defined by the specification as having "an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A" (see page 1, first sentence).

2.1.14. Yet additional subpopulations of hemolytic disease-suffering patients are also set forth in the application. For example, in the Examples (see page 27 et seqq.), which exemplify the successful treatment of patients afflicted with PNH, the patients so treated are defined by a number of clinical attributes: 18 years of age or older, diagnosed as having PNH with a type III erythrocyte population of greater than or equal to 10%, and having had at least four transfusions in the 12 months prior to treatment with the antagonist anti-C5 antibody eculizumab.

2.1.15. The skilled person would have also immediately appreciated another clinical feature of these patients: namely that "all patients were vaccinated against *Neisseria meningitides*" (see page 27, line 15). A mind willing to understand would understand that the inventors believed that all patients being treated with a complement inhibitor should be vaccinated and, thus, such action was not meant to be limited to a single example given in the application. In fact, there is no example presented in which a patient was not vaccinated. Also, there is no language in the exemplification at page 27 to indicate that use of the vaccine was meant to be limited to that example. As such, and referring to the criteria developed in the Guidelines as regards generalization, neither the general part of the application nor the specific example describes the feature to be inextricably linked to or related to the other features of said example, i.e. requirement a) as laid out in the Guidelines is met (see section 2.1.8, *supra*). Following therefrom, the feature is neither explained as essential or as indispensable for the function of the invention nor does the replacement or removal require a real modification of other features to compensate for the

change. Thus, also requirement b) as laid out in the Guidelines is met (see section 2.1.8, *supra*). Moreover, also requirement c) as laid out in the Guidelines is met (see section 2.1.8, *supra*), because as explained herein above, the invention relates to the treatment of patients afflicted with hemolytic disease and vaccination is a further measure to ensure treatment, as increased frequency of neisserial infections were observed in said patients. As said increased frequency is only related to the disease and not other parameters a generalizing isolation is justified, moreover so, as the specification is silent as to any limitation to specific embodiments. At least for these reasons, the introduction of the objected feature represents no undue generalization.

2.1.16. Thus, as elsewhere in the specification, the Examples clearly and unambiguously define characteristics of a novel patient population that the inventors believed would benefit, and did benefit, from treatment with an antagonist anti-C5 antibody. Additional patient population characteristics are presently recited in the instant claims, e.g., see claims 20, 22, and 23 of the enclosed claim set. And, as a practical matter, other than an objection for being based on a rejected base claim, the Office does not appear to contest the absolute support for these defined patient populations. Applicant's independent claims simply expressly articulate characteristics of a fully-defined and -described patient population set forth in the specification.

2.1.17. For the avoidance of doubt, this is not a case where an attempt has been made to extrapolate a general feature from a *specific embodiment* set forth in the application, as alleged by the Examiner. That is, unlike the subject claim in T157/90, for example, in which one embodiment alone, glycine, was provided as support for a broader genus of any additional amino acid, Applicant in this case points to a feature (a class of patients defined by their vaccination status) specifically recited in the application as the supportive basis for the same feature of the same scope recited in the claims. In other words, Applicant has merely taken a fully supported and manifestly clear limitation already existing *in haec verba* in the application and incorporated that limitation into the general invention now claimed. Applicant respectfully submits that a skilled person at the effective filing date of the application would have found such claims directly and unambiguously derivable from that previously presented by the application,

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which, as stated above, is the requirement for sufficient support according to the Guidelines.

2.1.18. Accordingly, it is respectfully requested that the objection to claims 1 to 3 is withdrawn.

2.2. Claim 4

2.2.1. Claim 4 is objected to as allegedly being in violation of Article 123(2) EPC because the expression used in the claim "altered constant region that exhibits decreased effector function relative to the corresponding native constant region" is allegedly not equivalent to the description, in which "the decreased effector function is said to be relative to an anti-CDCP1 antibody with a native constant region." Applicant traverses.

2.2.2. As noted above, the Technical Board of Appeal stated that, when reading a specification, interpretations which are illogical or which do not make technical sense should be ruled out. The reader should try, with synthetical propensity i.e. building up rather than tearing down, to arrive at an interpretation of the claim which is technically sensible and takes into account the whole disclosure of the patent. The patent must be construed by a mind willing to understand the invention (see T0190/99, loc. cit.). Applicant respectfully submits that the skilled person would immediately appreciate that the recitation of an "anti-CDCP1 antibody" is a simple typographical error in place of which an "anti-C5 antibody" was intended. Whereas the application recites "an anti-C5 antibody" exhaustively, nowhere else does the CDCP1 antibody appear in the document. The skilled person would immediately and directly understand that the "antibody that binds C5...[that] comprises an altered constant region....that exhibits decreased effector function" would be relative to the same antibody having a native constant region. This understanding is further underlined by the general knowledge in the art of antibody engineering[1] and the skilled person's

[1] *See*, e.g., WO 2007/084321 at page 42, second full paragraph stating "[a] native sequence Fc or constant region comprises an amino acid sequence identical to the amino acid sequence of a Fc or constant chain region found in nature. A variant or altered Fc or constant region comprises an amino acid sequence which differs from that of a native sequence heavy chain region by virtue of at least one amino acid modification, insertion, or deletion, for example. In certain embodiments, the variant or altered constant region has at least one amino acid substitution, insertion, and/or deletion, compared to a native sequence constant region or to the constant region of a parent polypeptide".

understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18;see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as **Exhibit A**)). Applicant also provides Mueller et al. (1997) *Mol Immunol* 34(6):441-452 as **Exhibit B**, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9).

2.2.3. Thus, claim 4 does not result in the skilled person being presented with information which is not directly and unambiguously derivable from that previously presented by the application, particularly when account is taken of matter which is implicit to said skilled person and the passage is read in the context of the application as a whole. Accordingly, reconsideration and withdrawal of the objection to claim 4 are respectfully requested.

2.3. Claim 6

2.3.1. Claim 6 was objected to as allegedly violating Article 123(2) EPC because the "dosage regimen given on page 22, lines 13-17 only refers to administration of the antibody and not to the one of the C5-binding fragment as claimed."

2.3.2. While not conceding to any aspect of the Examining Division's stated reasons for objection, previous claim 6 has been amended. As amended, the claim specifies that an anti-C5 antibody is to be administered to the patient under the specific dosage regimen recited. It is submitted that such an amendment renders the objection moot and therefore respectfully requests reconsideration and withdrawal of the objection to claim 6.

2.4. Claims 1 to 23

2.4.1. The Examiner notes with regard to the claims that "it is not possible to combine features taken from different independent embodiments or from the examples without violating the requirements of Article 123(2) EPC".

2.4.2. As noted above, neither the Guidelines nor the case law articulates an Article 123(2) EPC analysis that is as inflexible as that set forth in the Office Action. For example, in decision T1241/03 the Board denied that a claim "need[s] to have a literal basis in a single passage of the application as originally filed" to comport with Article 123(2) EPC (see T1241/03, Reasons for the Decision, paragraph 7). In T1241/03, the claims at issue were drawn to stable, aqueous liquid pharmaceutical formulations comprising human growth hormone (hGH) and a buffer providing pH in the range of 5.5 to 7, 0.1 to 1% by weight of a non-ionic surfactant and a neutral salt (T1241/03, Reasons for the Decision, paragraph 4). The claims were challenged as violating Article 123(2) because "the application as originally filed did not explicitly disclose the claimed formulation" in a single passage (T1241/03, Reasons for the Decision, paragraph 5). The Board rejected this challenge noting, among other things, that a general description of the claimed formulations and methods for producing stable formulations of hGH "by mixing it with a non-ionic surfactant in the range of 0.1 to 5% (w/v)" were expressly disclosed in the specification, albeit in separate passages (T1241/03, Reasons for the Decision, paragraph 6). The Board also noted that the working examples of the specification exemplified the generation and characterization of a stable formulation embraced by the claims. In view of this, the subject claims did "not refer to a 'patchwork' of parameters disclosed in non-connected parts of the description, nor [had] specific values been isolated from examples in a non-allowable way" (T1241/03, Reasons for the Decision, paragraph 7). Quite the contrary, the Board stated that considering the general disclosure of the application as filed, the reference in a claim to a combination of compounds in specific concentrations, explicitly disclosed in different passages of the application, is not considered to be an amendment of the patent which extends beyond the content of the application as originally filed.

2.4.3. Moreover, in another decision the Technical Board of Appeal has cautioned against Article 123(2) EPC determinations based on overly narrow readings of isolated passages in an as-filed specification. According to the Board, the question whether or not the instantly claimed subject matter is disclosed in the application as originally filed must take account of the whole content thereof and

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cannot solely be based on isolated parts of the description (see T823/96, Reasons for the Decision, paragraph 4).

2.4.4. In view of these legal principles, Applicant respectfully submits that the instant claims sufficiently satisfy the requirements of Article 123(2) EPC. The instant claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The anti-C5 antibody inhibits cleavage of complement component C5 into fragments C5a and C5b. The claims also require that the patients be vaccinated against *Neisseria meningitides* (e.g., claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (e.g., claims 2 and 3).

2.4.5. The claimed compositions (the single unit dosage forms) find full support, e.g., in the specification at page 5, lines 3 to 9. Further unequivocal support for administering such compositions to patients with hemolytic diseases is set forth in the same passage as well as at page 8, lines 19 to 21 and page 22, lines 7 to 17. And as noted above, support – particularly when viewed in the context of the general knowledge in the art and the entire teachings of the specification – for the treatment of a population of patients vaccinated against *Neisseria meningitides* is provided at page 27, line 15. In fact, the claims define characteristics of the patient population actually treated by the inventors.

2.4.6. Therefore, contrary to the position taken by the Office Action, it would have been clear to a skilled person that the instant specification sufficiently described the treatment of vaccinated patients afflicted with a hemolytic disease using the compositions as claimed. Like the claim upheld in T1241/03, it is clear that claims 1 to 3 do not refer to a 'patchwork' of parameters disclosed in non-connected parts of the description, nor have specific values been isolated from examples in a non-allowable way. Rather the specification provides literal support for each element of the claimed uses and instructive and supportive language that unambiguously tethers these elements together. Thus, in the words of the Board of Appeal, "considering the general disclosure of the application as filed, the reference in a claim to a combination of compounds ...

explicitly disclosed in different passages of the application, [should not be] considered...an amendment of the [claims] which extends beyond the content of the application as originally filed." Accordingly, as instant claims 1 to 3 (and all claims dependent therefrom) are directly and unambiguously derivable from the specification as filed, reconsideration and withdrawal of the rejection under Article 123(2) EPC are respectfully requested.

2.5. Dependent claims

2.5.1. Lastly, the Office Action also objects to all dependent claims under Article 123(2) as being based on independent claims allegedly in violation of the provision.

2.5.2. Applicant respectfully submits that for the reasons of record and those provided herein, the instant independent claims, and all claims dependent therefrom, satisfy the requirements of Article 123(2) EPC. Accordingly, reconsideration and withdrawal of this objection are respectfully requested.

3 UNITY (ARTICLE 82 EPC)

3.1. The Examining Division insinuates that in view of the previous line of arguments relating to the presence of three solutions to the posed problems the claims could be seen to lack unity.

3.2. As noted above, the instant claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The novel compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The claims also require that the patients be vaccinated against *Neisseria meningitides* (e.g., claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (e.g., claims 2 and 3).

3.3. Applicant respectfully submits that the Examining Division has at least constructively acknowledged that such claims – including the linking concepts of

the particular compositions and the use in conjunction with a meningitis vaccine or in vaccinated patients – are novel over the prior art. The instant claims are also inventive over that art for the reasons of record and those that follow.

4 INVENTIVE STEP (ARTICLE 56 EPC)

4.1. In section 3 of the Communication, the Examining Division maintains its inventive step objection over D1 in combination with D5 raised in the ESOP which follows the problem-solution-approach. According to the Examining Division, Applicant's prior arguments in favor of inventiveness were not "appropriate in that they [allegedly] focus on the differences between D1 or D5 and the claimed invention....the complementary teachings of D1 and D5 have been combined for assessing the inventiveness of the invention."

4.2. Applicant cannot follow the Examining Division's assessment. The claimed invention is based, at least in part, upon Applicant's discovery that during an early clinical trial evaluating eculizumab as a safe and effective therapy, a patient undergoing complement suppressive therapy developed a meningitis infection. In view of this previously unrecognized problem, and to further safeguard the well-being of PNH patients in need of eculizumab therapy, Applicant affirmatively chose to treat PNH patients with an anti-C5 antibody only after the patients had been vaccinated against *Neisseria meningitides*, or where the antibody was used in conjunction with such a vaccination. The instant claims embrace this inventive concept. Claim 1, for example, is drawn to the use of anti-C5 antibody-containing composition for treating PNH patients who have been vaccinated against *Neisseria*. Claims 2 and 3 are drawn to embodiments in which the anti-C5 antibody-containing composition is to be therapeutically administered to PNH patients in conjunction with vaccination.

4.3. Whether alone, or in combination, the cited references fail to disclose or suggest such uses.

4.4. D5, which *per se* fails to disclose the instantly claimed pharmaceutical composition unit dosage forms, does not relate to hemolytic disease at all, but

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rather methods for treating, among other things, rheumatoid arthritis. And although the methods of D5 can include the use of an anti-C5 antibody, there is no disclosure or even a suggestion in D5 of using the claimed compositions in patients that have been vaccinated against *Neisseria meningitides*. Therefore, D5 does not deprive the instant claims of inventiveness. However, the Communication appears to assert that there would have been a reason to combine the teachings of D5 with that of D1 to thereby arrive at the instantly claimed uses. Applicant submits that this is not the case. In fact, absent the teachings of the specification, there would have been no reason at all for a skilled artisan to practice the uses now claimed.

4.5. D1 teaches, among other things, methods for treating PNH using an anti-C5 antibody. As acknowledged by the Examining Division, however, the reference does not disclose pharmaceutical compositions comprising a single unit dosage form of 300 mg of an anti-C5 antibody. D1 does make one reference to meningitis at paragraph [0041], which characterizes the general knowledge in the art at the time as being that "patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone". However, this passage alone would not have provided the requisite inertia for a skilled person to, first, make a composition neither described nor suggested in D1 or D5, and then use such a composition in conjunction with a meningitis vaccine or in vaccinated patients. Only with the guidance provided by the inventors in the instant specification would there have been a reason for a skilled person at the effective filing date of the application to arrive at the instantly claimed uses.

5 REQUESTS

With the above amendments and explanations it is submitted that the Applicant has met the requirements as set forth in the Communication. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

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If, however, the Examining Division does not agree with the above, it is requested that either a further Communication pursuant to Art. 94(3) EPC and Rule 71(2) EPC or a summons to attend oral proceedings according to Art. 116(1) EPC be issued. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure
New claims 1 to 23
Exhibit A and B

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Page 585

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URGENT! PLEASE FORWARD IMMEDIATELY TO THE EXAMINING DIVISION!
ORAL PROCEEDINGS SCHEDULED FOR SEPTEMBER 5, 2019

September 2, 2019

Third Party Observations

Application Number	16 195 355.9 - 1466
Publication Number	EP 3 167 888
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Applicant	Alexion Pharmaceuticals, Inc.
Our file reference	AMG17203ADV-EP888

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

The Applicant acted against the well-established principle of *nemo potest venire contra factum proprium*:

(1) In their submission dated August 15, 2018, Alexion attempt to defend their contradictive behavior in the proceedings regarding EP 1 720 571 and the current application. The Applicant's representative asserts that the principle of *nemo potest venire contra factum proprium* would not be applicable in this case (cf. p. 6, ultimate par. of submission dated August 15, 2018). This is incorrect.

(2) The principle of the protection of legitimate expectations is a general principle well established in European Community law and generally recognized in the EPC contracting states and Board of Appeal case law (**G 2/97**, OJ 1999, 123; see also **R 4/09**). The term "good faith" is also used to describe this concept (**J 10/84**, OJ 1985, 71; **J 38/97**). Hence, it is established case law that users of the European patent system, who are

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parties in proceedings before the EPO, must act in good faith (**G 2/97**, **R 4/09**).

(3) According to the "Saxe Declaration" submitted by the Applicant on January 16, 2018, Stephen Saxe submitted a letter to CAS dated April 28, 2009 explaining:

> *"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."*

(4) Thus, Alexion were aware that they provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Contrary to the Applicant's assertion, when filing the response of December 30, 2013 in the EP'571 case, Alexion thus did not make a *"mere assertion that turned out to be erroneous"* (cf. p. 6, 4th par. of submission dated August 15, 2018). Rather, they made an assertion and at the same time apparently assumed that the contrary was the case.

(5) In their submission dated July 5, 2019, Alexion argue that the copy of the CAS entry, provided with their letter in the opposition proceedings regarding EP 1 720 571 did not contain any sequences, that the identity of the sequences was not investigated, and that the argument was not challenged at that time (cf. p. 6, 6th to 9th par. of letter dated July 5, 2019). We note that none of these allegations take away from the fact that Alexion have asserted before the OD in the EP'571 case that the sequence of eculizumab had been submitted to CAS knowing that this was incorrect.

(6) Alexion further try to defend themselves by arguing that they did not gain any advantage from their knowingly false statement (cf. p. 7, 1st par. of letter dated July 5, 2019). Of course, the obligation to act in good faith does not depend on the question whether a party gains an advantage or not.

(7) On the other hand, Alexion did not provide any evidence that they indeed filed the wrong structural information to CAS, since the Saxe Declaration - contrary to the assertion of Alexion's representative - does not include said "wrong" structure or any excerpt of the CAS registry showing that a "wrong" structure was submitted there. With the letter dated July 5, 2019, Alexion filed an excerpt from a CAS online resource allegedly showing the sequences originally submitted to CAS (**D28**). We note that the document provided by the Applicant does not include any information about the date on which the sequence shown therein has been submitted to CAS and/or been amended. This document, apparently retrieved on June 11, 2019, is therefore

unable to prove that the incorrect sequence has been submitted to CAS in 1999, i.e., twenty years ago.

(8) In this regard we would like to point out that the Applicant's allegation – that the patents and applications cited in **D8** would refer to anti-C5 antibodies more generally and that it would, therefore, be of no importance whether or not the correct sequence of eculizumab had been disclosed (p. 8, 1st to 3rd full paragraph of Applicants submission of August 15, 2018) - is simply not tenable. Rather, the question whether or not the correct sequence of the antibody designated via the INN eculizumab was available at CAS – published and provided with a CAS registry number - was of utmost importance.

(9) An INN is an **official** generic and non-proprietary **name** given to an active ingredient. The INN "eculizumab" is the **official** generic **name** given to the substance having the CAS-Number 219685-50-4. In conclusion, the above presented facts inevitably imply that the public – in particular authors of scientific literature but also the EPO - could rely upon the fact that a correct description of eculizumab was publicly available at the priority date.

(10) As will be explained below, during prosecution of EP 1 720 571 B1 and EP 2 359 834 B1, the Examining Division considered the term "eculizumab" at all times to disclose the specific antibody related to said INN. Alexion's representatives took the same position, even though Alexion apparently assumed at least as of April 2009 that the INN eculizumab related to a false structure (as they allege now).

(11) Claim 1 of EP 1 720 571 B1 as granted directly relates to eculizumab. It is readily derivable from the prosecution history (see first office action dated September 2, 2009 (enclosed as **Annex I**), Items 4.1 to 4.3 of the Communication; Summons to Oral Proceedings dated March 4, 2011 (enclosed as **Annex II**), Items 2.1, 5.1, 6.1) that the Examining Division heavily relied upon the fact that eculizumab was known on the priority date. In the Summons to Oral Proceedings the Examining Division further stated under Item 3.2 that:

> *"The application provides experimental details of dosage optimization of an anti-C5 antibody, 5G1.1* ***eculizumab****, for the treatment of PNH in a group of 11 patients.* ***Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used****. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention."*

(12) Although Alexion assumed that eculizumab was not disclosed at the priority date, as they now allege, the disclosure of eculizumab was confirmed by them vis-à-vis the Examining Division (see reply to Summons to Oral Proceedings dated September 23, 2011 (enclosed as **Annex III**), page 9, second paragraph). Because the application as filed did not disclose the actual structure of eculizumab this was the only way to overcome the cited objection.

(13) The sole claim of EP 2 359 834 B1 as granted directly relates to eculizumab. Again, the Examining Division heavily relied upon the notion that eculizumab had been disclosed before the priority date (see e.g. European Search Opinion dated July 21, 2011 (enclosed as **Annex IV**), objection under Item 2, upheld by the Examining Division in further prosecution). The prior disclosure of eculizumab was also confirmed by Alexion *vis-à-vis* the Examining Division (see reply to Office Action dated November 15, 2012 (enclosed as **Annex V**), Item 2.2.2):

> *"This understanding is further underlined by the general knowledge in the art of antibody engineering and the skilled person's understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18; see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as Exhibit A)). Applicant also provides Mueller et al. (1997) Mol Immunol 34(6):441-452 as Exhibit B, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9)."* (Emphasis added.)

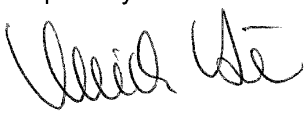
(14) Again, Alexion relied upon the public's knowledge of the structure of eculizumab before the Examining Division to support the patentability of claims to "eculizumab" but now assert that one of skill in the art lacked such knowledge.

(15) In summary, the WHO named eculizumab to be the official generic name given to the substance having the CAS-Number 219685-50-4. According to their own statements, Alexion were aware of having provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Therefore, the concealment of this information cannot be considered to merely have been erroneous. By concealing this information Alexion cannot be said to have acted in good faith when attempting to overcome the various objections from the Examining Division, which objections could not have been overcome had the EPO been informed that the INN eculizumab was in fact assigned to a false structure. On the other hand, there is no evidence provided that Alexion have indeed filed "wrong" structural information to CAS. At the moment, this appears to be a mere assertion.

(16) In conclusion, Alexion's conduct vis-à-vis the EPO is contrary to the principle of *nemo potest venire contra factum proprium*, and their attempt to justify their concealment of alleged facts, which, if true would have been critical for validity in a number of proceedings, and which are

nonetheless now supposed to serve as basis to restore novelty over prior art documents, must be refused.

Respectfully



Dr. H. Ulrich Dörries

Enclosures
Annexes I-V

Europäisches Patentamt
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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	AMG17203ADV-EP888

	Description of document	Original file name	Assigned file name
1	Observations by third parties (Art. 115 EPC)	TPO.pdf	TIPA1-1.pdf
2	Citations filed by a third party	Annex I - EP1720571_first office action_02.pdf	TDOC-1.pdf
3	Citations filed by a third party	Annex II - EP1720571_summons to oral proceedings_04.pdf	TDOC-2.pdf
4	Citations filed by a third party	Annex III - EP1720571_reply to summons to oral proceedings.pdf	TDOC-3.pdf
5	Citations filed by a third party	Annex IV - EP2359834_european search opinion_21.07.20111.pdf	TDOC-4.pdf
6	Citations filed by a third party	Annex V - EP2359834_reply to office action_16.pdf	TDOC-5.pdf

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Munich, Germany**
Date:	**02 September 2019**
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Representative name:	**H. Ulrich Dörries**
Capacity:	**(Representative)**

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	7819721
Application number	EP16195355.9
Date of receipt	02 September 2019
Receiving Office	European Patent Office, The Hague
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Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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WO 2004/035607 A2

(54) Title: HUMAN MONOCLONAL ANTIBODIES AGAINST CD20

(57) Abstract: Isolated human monoclonal antibodies which bind to and inhibit human CD20, and related antibody-based compositions and molecules, are disclosed. The human antibodies can be produced by a transfectoma or in a non-human transgenic animal, *e.g.*, a transgenic mouse, capable of producing multiple isotypes of human monoclonal antibodies by undergoing V-D-J recombination and isotype switching. Also disclosed are pharmaceutical compositions comprising the human antibodies, non-human transgenic animals and hybridomas which produce the human antibodies, and therapeutic and diagnostic methods for using the human antibodies.

HUMAN MONOCLONAL ANTIBODIES AGAINST CD20

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No.: 60/419163, filed on October 17, 2002, and U.S. Provisional Application No.: 60/460028, filed on April 2, 2003, both entitled HUMAN MONOCLONAL ANTIBODIES AGAINST CD20, and both of which are incorporated herein in their entirety by this reference.

BACKGROUND OF THE INVENTION

The CD20 molecule (also called human B-lymphocyte-restricted differentiation antigen or Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al.* (1989) *J. Biol. Chem.* 264(19):11282-11287; and Einfield *et al.* (1988) *EMBO J.* 7(3):711-717). CD20 is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs and is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. In particular, CD20 is expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson *et al.* (1984) *Blood* 63(6):1424-1433), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal tissues (Tedder *et al.* (1985) *J. Immunol.* 135(2):973-979).

The 85 amino acid carboxyl-terminal region of the CD20 protein is located within the cytoplasm. The length of this region contrasts with that of other B cell-specific surface structures such as IgM, IgD, and IgG heavy chains or histocompatibility antigens class II α or β chains, which have relatively short intracytoplasmic regions of 3, 3, 28, 15, and 16 amino acids, respectively (Komaromy *et al.* (1983) NAR 11:6775-6785). Of the last 61 carboxyl-terminal amino acids, 21 are acidic residues, whereas only 2 are basic, indicating that this region has a strong net negative charge. The GenBank Accession No. is NP_690605.

It is thought that CD20 might be involved in regulating an early step(s) in the activation and differentiation process of B cells (Tedder *et al.* (1986) *Eur.J. Immunol.* 16:881-887) and could function as a calcium ion channel (Tedder *et al.* (1990) *J. Cell. Biochem.* 14D:195).

Despite uncertainty about the actual function of CD20 in promoting proliferation and/or differentiation of B cells, it provides an important target for antibody mediated therapy to control or kill B cells involved in cancers and autoimmune disorders. In particular, the expression of CD20 on tumor cells, *e.g.*, NHL, makes it an important target for antibody mediated therapy to specifically target therapeutic agents

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against CD20-positive neoplastic cells. However, while the results obtained to date clearly establish CD20 as a useful target for immunotherapy, they also show that currently available murine and chimeric antibodies do not constitute ideal therapeutic agents.

Accordingly, the need exists for improved therapeutic antibodies against CD20 which are effective in preventing and/or treating a range of diseases involving cells expressing CD20.

SUMMARY OF THE INVENTION

The present invention provides improved antibody therapeutics for treating and/or preventing diseases associated with cells expressing CD20, including tumor-related diseases, and immune diseases, including autoimmune diseases. The antibodies encompassed by the invention are improved in that they are fully human and, thus, are potentially less immunogenic in patients.

As exemplified herein, the human antibodies of the invention mediate killing of B cells expressing CD20 by a variety of mechanisms. In one embodiment, human antibodies of the invention induce complement dependent cytotoxicity (CDC), *e.g.*, at least about 20% CDC mediated lysis, preferably about 30% CDC mediated lysis, and more preferably 40-50% mediated lysis in cells, such as chronic B-lymphocytic leukaemia (B-CLL) cells. In another embodiment, human antibodies of the invention induce apoptosis of cells expressing CD20. In another embodiment, human antibodies of the invention induce homotypic adhesion of cells expressing CD20. Furthermore, the human antibodies of the invention may induce antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20 in the presence of human effector cells (*e.g.*, monocytes, mononuclear cells, NK cells and PMNs). Furthermore, human antibodies of the invention may induce phagocytosis of cells expressing CD20 in the presence of macrophages. The human monoclonal antibodies of the invention may work by one or more of these mechanisms. Examples of cells which can be killed by human antibodies of the present invention include, but are not limited to, B cells expressing CD20, such as tumorigenic B cells and B cells involved in immune diseases. In a particular embodiment, the human antibodies are used to mediate killing of B lymphocytes in the treatment of lymphoma, *e.g.*, B cell non-Hodgkin's lymphoma.

Human antibodies of the invention include IgG1 (*e.g.*, IgG1,κ), IgG3 (*e.g.*, IgG3,κ) and IgG4 (*e.g.*, IgG4,κ) antibodies. However, other antibody isotypes are also encompassed by the invention, including IgG2, IgM, IgA1, IgA2, secretory IgA, IgD, and IgE. The antibodies can be whole antibodies or antigen-binding fragments thereof including, for example, Fab, $F(ab')_2$, Fv, single chain Fv fragments or bispecific antibodies. Furthermore, the antigen-binding fragments include binding-domain

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immunoglobulin fusion proteins comprising (i) a binding domain polypeptide (such as a heavy chain variable region or a light chain variable region) that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. Such binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939.

Particular human antibodies of the present invention include those referred to as 11B8, 2F2, and 7D8, encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof. In another embodiment, the human antibodies are characterized by having human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

In yet another embodiment, the human antibodies are characterized by having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively; SEQ ID NO:6 and SEQ ID NO:8, respectively; or SEQ ID NO:10 and SEQ ID NO:12, respectively.

Other particular human antibodies of the invention include those which comprise a CDR domain having a human heavy and light chain CDR1 region, a human heavy and light chain CDR2 region, and a human heavy and light chain CDR3 region, wherein

(a) the CDR1, CDR2, and CDR3 human heavy chain regions comprise an amino acid sequence selected from the group consisting of the amino acid sequences CDR1, CDR2, and CDR3 shown in Figures 53, 55, or 57 (SEQ ID NOs:13-15, 19-21, and 25-27), and conservative sequence modifications thereof, and

(b) the CDR1, CDR2, and CDR3 human light chain regions comprise an amino acid sequence selected from the group consisting of the amino acid sequences CDR1, CDR2, and CDR3 shown in Figures 53, 55, or 57 (SEQ ID NOs: 16-18, 22-24, and 28-30), and conservative sequence modifications thereof.

Also included within the present invention are antibodies which dissociate from CD20 with a dissociation equilibrium constant (K_D) of approximately 1-10 nM or less. Such antibodies also include those which do not cross-react with related cell-surface antigens and thus do not inhibit their function.

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In another embodiment, human anti-CD20 antibodies of the present invention can be characterized by one or more of the following properties:

a) specificity for human CD20;

b) a binding affinity to CD20 (K_D) of about 10 nM or less, preferably, about 5 nM or less and, more preferably, about 1-3 nM or less as determined by the binding experiment disclosed in Example 5 (Figure 9) herein;

c) a dissociation rate constant (k_d) from CD20 of about 10^{-4} sec^{-1} or less, preferably, about 10^{-5} sec^{-1} or less and, more preferably, about 10^{-6} sec^{-1} or less, as determined by the dissociation rate experiment disclosed in Example 5 (Figure 9) herein;

d) the ability to mediate a high level of CDC on either CD55/59 negative or CD55/59 positive cells;

e) the ability to translocate into lipid rafts upon binding to CD20;

f) the ability to inhibit the growth of cells which express CD20;

g) the ability to induce apoptosis of cells which express CD20;

h) the ability to induce homotypic adhesion of cells which express CD20;

i) the ability to induce ADCC of cells which express CD20 in the presence of effector cells;

j) the ability to prolong survival of a subject having tumor cells which express CD20;

k) the ability to deplete cells which express CD20; and/or

l) the ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

The human anti-CD20 antibodies of the present invention can be derivatized, linked to or co-expressed to other binding specificities. In a particular embodiment, the invention provides a bispecific or multispecific molecule comprising at least one first binding specificity for CD20 (*e.g.*, a human anti-CD20 antibody or mimetic thereof), and a second binding specificity for a human effector cell, such as a binding specificity for an Fc receptor (*e.g.*, a human Fcγ receptor, such as FcγRI, or a human Fcα receptor) or a T cell receptor, *e.g.*, CD3.

Accordingly, the present invention includes bispecific and multispecific molecules that bind to both human CD20 and to an Fc receptor or a T cell receptor, *e.g.*, CD3. Examples of Fc receptors are, *e.g.*, a human IgG receptor, *e.g.*, an Fc-gamma receptor (FcγR), such as FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). Other Fc receptors, such as human IgA receptors (*e.g.*, FcαRI), also can be targeted. The Fc receptor is preferably located on the surface of an effector cell, *e.g.*, a monocyte, macrophage or an activated mononuclear cell. In a preferred embodiment, the bispecific and multispecific molecules bind to an Fc receptor at a site which is distinct from the immunoglobulin Fc (*e.g.*, IgG or IgA) binding site of the receptor. Therefore, the

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binding of the bispecific and multispecific molecules is not blocked by physiological levels of immunoglobulins.

In yet another aspect, human anti-CD20 antibodies of the invention are derivatized, linked to or co-expressed with another functional molecule, *e.g.*, another peptide or protein (*e.g.*, a Fab' fragment). For example, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, to produce a bispecific or a multispecific antibody), a cytotoxin, cellular ligand or antigen (*e.g.*, to produce an immunoconjugate, such as an immunotoxin). An antibody of the present invention can be linked to other therapeutic moieties, *e.g.*, a radioisotope, a small molecule anti-cancer drug, an anti-inflammatory agent, or an immunosuppressive agent. Accordingly, the present invention encompasses a large variety of antibody conjugates, bispecific and multispecific molecules, and fusion proteins, all of which bind to CD20 expressing cells and which can be used to target other molecules to such cells.

In still another aspect, the invention provides compositions, *e.g.*, pharmaceutical and diagnostic compositions/kits, comprising a pharmaceutically acceptable carrier formulated along with one or a combination of human monoclonal antibodies of the invention. In a particular embodiment, the composition includes a combination of antibodies which bind to distinct epitopes or which possess distinct functional characteristics, such as inducing CDC and inducing apoptosis.

Human antibodies, immunoconjugates, bispecific and multispecific molecules and compositions of the present invention can be used in a variety of methods for inhibiting growth of cells expressing CD20 and/or killing cells expressing CD20 by contacting the cells with an effective amount of the antibody, immunconjugate, bispecific/multispecific molecule or composition, such that the growth of the cell is inhibited and/or the cell is killed. In one embodiment, the method includes killing of the cell expressing CD20 in the presence of effector cells, for example, by CDC, apoptosis, ADCC, phagocytosis, or by a combination of two or more of these mechanisms. The cells are preferably killed or inhibited without killing or inhibiting the activity of cells which do not express CD20 but which may, for example, express a structurally related cell-surface antigen (*i.e.*, without cross-reactivity to related but functionally distinct cell surface antigens). Cells expressing CD20 which can be inhibited or killed using the human antibodies of the invention include, for example, tumorigenic B cells.

Accordingly, human antibodies of the present invention can be used to treat and/or prevent a variety of diseases involving cells expressing CD20 by administering the antibodies to patients suffering from such diseases. Exemplary diseases that can be treated (*e.g.*, ameliorated) or prevented include, but are not limited

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to, tumorigenic diseases and immune diseases, *e.g.*, autoimmune diseases. Examples of tumorigenic diseases which can be treated and/or prevented include B cell lymphoma, *e.g.*, NHL, including precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), including low-grade, intermediate-grade and high-grade FL, cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL). Examples of immune disorders in which CD20 expressing B cells are involved which can be treated and/or prevented include psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases. Further examples are severe acute respiratory distress syndrome and choreoretinitis. Yet further examples are diseases and disorders caused by infection of B-cells with virus, such as Epstein-Barr virus (EBV).

In a particular embodiment of the invention, the subject being administered the antibody is additionally treated with a chemotherapeutic agent, radiation, or an agent that modulates, *e.g.*, enhances or inhibits, the expression or activity of an Fc receptor, *e.g.*, an Fcα receptor or an Fcγ receptor, such as a cytokine. Typical cytokines for administration during treatment include granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF). Typical therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide.

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In yet another aspect, the present invention provides a method for detecting *in vitro* or *in vivo* the presence of CD20 in a sample or individual, *e.g.*, for diagnosing a CD20-related disease, preferably at an early stage. This can also be useful for monitoring the disease and effect of treatment and for determining and adjusting the dose of the antibody to be administered. The *in vivo* method can be performed using imaging technique such as PET (positron emission tomography) or SPECT (single photon emission computed tomography). In one embodiment, this is achieved by contacting a sample to be tested, optionally along with a control sample, with a human monoclonal antibody of the invention under conditions that allow for formation of a complex between the antibody and CD20. Complex formation is then detected (*e.g.*, using an FACS analysis or Western blotting). When using a control sample along with the test sample, complex is detected in both samples and any statistically significant difference in the formation of complexes between the samples is indicative of the presence of CD20 in the test sample.

In yet another aspect, the invention provides a transgenic non-human animal, such as a transgenic mouse, which express human monoclonal antibodies that bind to CD20. In a particular embodiment, the transgenic non-human animal is a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention. The transgenic non-human animal can be immunized with a purified or enriched preparation of CD20 antigen and/or cells expressing CD20. Preferably, the transgenic non-human animal, *e.g.*, the transgenic mouse, is capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgM) by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

Accordingly, in yet another aspect, the invention provides isolated B cells from a transgenic non-human animal as described above, *e.g.*, a transgenic mouse, which expresses human anti-CD20 antibodies. The isolated B cells can then be immortalized by fusion to an immortalized cell to provide a source (*e.g.*, a hybridoma) of human anti-CD20 antibodies. Such hybridomas (*i.e.*, which produce human anti-CD20 antibodies) are also included within the scope of the invention.

As exemplified herein, human antibodies of the invention can be obtained directly from hybridomas which express the antibody, or can be cloned and recombinantly expressed in a host cell (*e.g.*, a CHO cell, a NS/0 cell or a lymphocytic cell). Further examples of host cells are microorganisms, such as *E. coli*, and fungi, such as yeast. Alternatively, they can be produced recombinantly in a transgenic non-human animal or plant. Accordingly, in another aspect, the present invention provides methods for producing human monoclonal antibodies which bind to human CD20. In

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one embodiment, the method includes immunizing a transgenic non-human animal, *e.g.*, a transgenic mouse, as previously described (*e.g.*, having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an anti-CD20 antibody), with a purified or enriched preparation of human CD20 antigen and/or cells expressing human CD20. B cells (*e.g.*, splenic B cells) of the animal are then obtained and fused with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies against CD20.

In yet another aspect, the invention provides nucleic acid molecules encoding human anti-CD20 antibodies (*e.g.*, variable regions thereof), as well as recombinant expression vectors which include the nucleic acids of the invention, and host cells transfected with such vectors. Methods of producing the antibodies by culturing these host cells are also encompassed by the invention. Particular nucleic acids provided by the invention comprise the nucleotide sequences shown in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, encoding the heavy and light chains, respectively, of human anti-CD20 antibodies 2F2, 7D8, and 11B8.

Other features and advantages of the instant invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the pCONγ1f/variable-heavy vector used for recombinant production of the human monoclonal antibodies 2F2 and 11B8.

Figure 2 shows the pCONκ/variable-light vector used for recombinant production of 2F2 and 11B8.

Figure 3 shows the double-gene cloning vector (pCONγ1f/κ2F2) used for recombinant production of 2F2 and 11B8.

Figure 4 is a graph comparing the binding of human monoclonal antibodies 2F2, 7D8, and 11B8 to Raji, Daudi, and CD20 transfected NS/0 cells and parental NS/0 cells using flow cytometry.

Figures 5A and 5B show the binding of 2F2 to PBMCs from three human donors using flow cytometry.

Figure 6 is a graph comparing the binding affinity of ^{125}I-labeled 2F2 and ^{125}I-labeled 11B8 to Ramos-EHRB cells.

Figures 7A and 7B show the binding of ^{125}I-labeled 2F2 and ^{125}I-labeled 11B8 compared to ^{125}I-labeled rituximab (chimeric anti-CD20 antibody, IDEC) and ^{125}I-labeled B1 (the term B1 corresponds to the unlabeled form of Bexxar™, which is a ^{131}I-labeled murine anti-human CD20 antibody, Coulter) to Ramos-EHRB cells (A) and Daudi cells (B).

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Figure 8 is a graph comparing the dissociation rates of ^{125}I-labeled 11B8T, ^{125}I-labeled 2F2, ^{125}I-labeled rituximab (RIT), and ^{125}I-labeled B1.

Figure 9 shows the dissociation rates of the F(ab')$_2$ fragments of 2F2, 11B8T, and rituximab in Ramos-EHRB cells.

Figures 10A and 10B show the CDC by 2F2T, 11B8T, 7D8, rituximab, and an isotype control antibody (HuMab-KLH) of Daudi cells (A) and SU-DHL-4 cells (B) at different time points (functional off-rate) using flow cytometry.

Figures 11A-E show the kinetics of CDC induced by 2F2 and rituximab in different cell lines using flow cytometry.

Figures 12A-D show CDC induced by 2F2 and rituximab in different cell lines as a function of the concentration of complement (normal human serum (NHS)) at two different antibody concentrations using flow cytometry.

Figures 13A-D show concentration-dependent induction of CDC by 2F2 and rituximab in different cell lines using flow cytometry.

Figures 14A and 14B show concentration-dependent induction of CDC by 2F2, 2F2T, 11B8T, B1, and rituximab in Daudi cells (A) and Raji cells (B).

Figures 15A and 15B are graphs comparing CDC of Daudi cells (cells expressing low levels of CD55/59) by human monoclonal antibodies 2F2, 7D8, and 11B8 and rituximab; (A) shows percent lysis of unwashed cells and (B) shows percent lysis of cells which were washed before the addition of serum.

Figures 16A and 16B are graphs comparing CDC of Raji cells (cells expressing high levels of CD55/59) by human monoclonal antibodies 2F2, 7D8, and 11B8 and rituximab; (A) shows percent lysis of cells not blocked with anti-CD55 and anti-CD59 antibodies, and (B) shows percent lysis of cells blocked with anti-CD55 and anti-CD59 antibodies.

Figures 17A-C show the role of CD55 and CD59 in CDC induced by 2F2 and rituximab in Raji cells. (A) shows the percentage of lysed cells upon addition of anti-CD55 antibody, (B) shows the percentage of lysed cells upon addition of anti-CD59 antibody, and (C) shows the percentage of lysed cells upon addition of both anti-CD55 antibody and anti-CD59 antibody.

Figures 18A-D show the binding of complement factor C1q by 2F2 and rituximab in different cell lines as determined by flow cytometry.

Figures 19A-D show the deposition of complement factor fragment C4c by 2F2 and rituximab in different cell lines as determined by flow cytometry.

Figure 20 shows lysis of ARH-77 cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 21 shows lysis of B-CLL cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

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Figure 22 shows lysis of HCL (hairy cell leukemia) cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 23 shows lysis of B-ALL cells by 2F2, and rituximab, in the presence of PMNs, MNCs, plasma or whole blood.

Figure 24 shows lysis of follicular lymphoma (FL) cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 25 shows lysis of mantle cell lymphoma cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 26 shows concentration dependent lysis of ARH-77 cells by 2F2 and rituximab in the presence of whole blood.

Figure 27 shows MNC-mediated lysis of ARH-77 cells by 2F2T, 11B8T, and rituximab.

Figure 28 shows MNC-mediated lysis of Raji cells by 2F2T, 11B8T, and rituximab.

Figures 29A, B, and C are graphs showing clustering of CD20 in the lipid rafts upon incubation with 2F2, 7D8, or 11B8 using FRET analysis and Triton-X insolubility assay.

Figure 30 shows clustering of CD20 in the lipid rafts upon incubation with 2F2, rituximab, or 11B8T using FRET analysis.

Figure 31 shows the proportion of CD20 remaining in the insoluble raft fraction after treatment with Triton X-100 (TX) and incubation with 2F2, rituximab, or 11B8T.

Figure 32 shows the distribution of CD20 between the raft and non-raft membrane fractions upon stimulating Daudi cells with 2F2, rituximab, or 11B8T.

Figures 33A-G show apoptosis of Daudi cells by 2F2, 7D8, and 11B8 using flow cytometry.

Figure 34 shows induction of apoptosis of Raji cells by 2F2, 11B8T, rituximab, or B1 using flow cytometry.

Figure 35A shows induction of apoptosis of Daudi cells by 2F2T, 11B8T, rituximab, or B1 using flow cytometry.

Figure 35B shows early stage and late stage apoptosis of Daudi cells by human monoclonal antibodies 2F2T, 11B8T, rituximab, and B1 using flow cytometry.

Figures 36A-E show homotypic adhesion of Ramos-EHRB cells by 2F2, 7D8, and 11B8 using light microscopy.

Figure 37 show homotypic adhesion of Daudi cells by 2F2, rituximab, and B1 using light microscopy.

Figure 38 is a graph showing the percent survival of SCID mice injected with Daudi cells and treated with 2F2 or 7D8.

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Figure 39 shows the percent survival of SCID mice injected with Tanoue cells and treated with 2F2, rituximab, or B1.

Figure 40 shows the percent survival of SCID mice injected with Daudi cells and treated with different concentrations of 2F2 or rituximab.

Figure 41 shows the percent survival of SCID mice injected with Daudi cells and treated with 11B8T or B1.

Figure 42 shows bioluminescence imaging of tumor cells in SCID mice on day 39 (31 days after treatment with 10 μg of B1, rituximab, 11B8T, 2F2T, or huIgG1). The bioluminescence is represented in red color (the dark areas in the mice) (light intensity > 50 photons per 5 min) as overlay on the black and white body image of the mice.

Figure 43 shows the tumor mass in each mouse quantified on day 25, 32, 39, and 46 following administration, on day 8, of 10 μg of B1, rituximab, 11B8T, 2F2T, or huIgG1 by integrating the light signals over the body surface.

Figures 44A-C show flow cytometric analysis of $CD20^+$ cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 45A-C show flow cytometric analysis of $CD21^+$ cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 46A-C show flow cytometric analysis of $CD20^+$ cells in lymph node of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 47A-C show flow cytometric analysis of $CD20^{low}CD23^+CD40^{high}$ expressing cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 48A-E show binding of rituximab (A), 2F2 (B), 11B8 (C), B1 (D), or an isotype control antibody (E) to CHO cells expressing wild type (WT) CD20, mutant CD20 (AxP), or both WT CD20 and mutant CD20 (AxP) as determined by flow cytometry.

Figures 49A-F show percentage binding of 2F2, 11B8T, B1 or rituximab to mutant P172S vs. WT CD20 (A), percentage binding of 2F2T, 11B8T, B1, CAT (CAT 13.6E12, a mouse monoclonal IgG2A anti-CD20 antibody, Diatec.Com), a control isotype antibody (KLH) or rituximab to mutant CD20 (AxP) vs.

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WT CD20 (B), percentage binding of 2F2, 11B8T, B1 or rituximab to mutant N166D vs. WT CD20 (C), percentage binding of 2F2T, CAT or rituximab to mutant N166D vs. WT CD20 (D) , percentage binding of 2F2T, 2F2, 11B8T, B1 or rituximab to mutant N163D vs. WT CD20 (E), and percentage binding of 2F2T, CAT or rituximab to mutant N163D vs. WT CD20 (F).

Figure 50 shows binding of 2F2T, 7D8, and isotype control antibody, as determined by ELISA, to three anti-idiotypic antibodies, anti-2F2 sab 1.1, anti-2F2 sab 1.2, and anti-2F2 sab 1.3, raised against 2F2.

Figure 51 shows binding of 11B8T, as determined by ELISA, to anti-idiotypic antibodies, anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6, raised against 11B8T, but no binding to the anti-idiotypic anti-2F2 antibodies.

Figures 52A-C show dose-dependent binding of 2F2T, as determined by ELISA, to three anti-idiotypic antibodies, anti-2F2 sab 1.1 (A), anti-2F2 sab 1.2 (B), and anti-2F2 sab 1.3 (C), raised against 2F2.

Figure 53 shows the amino acid sequence (SEQ ID NO:2) of the heavy chain V region and the amino acid sequence (SEQ ID NO:4) of the light (kappa) chain V region of human monoclonal antibody 2F2 with CDR regions designated.

Figure 54 shows the nucleotide sequence (SEQ ID NO:1) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:3) of the light (kappa) chain V region of human monoclonal antibody 2F2.

Figure 55 shows the amino acid sequence (SEQ ID NO:6) of the heavy chain V region and the amino acid sequence (SEQ ID NO:8) of the light (kappa) chain V region of human monoclonal antibody 7D8 with CDR regions designated.

Figure 56 shows the nucleotide sequence (SEQ ID NO:5) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:7) of the light (kappa) chain V region of human monoclonal antibody 7D8.

Figure 57 shows the amino acid sequence (SEQ ID NO:10) of the heavy chain V region and the amino acid sequence (SEQ ID NO:12) of the light (kappa) chain V region of human monoclonal antibody 11B8 with CDR regions designated.

Figure 58 shows the nucleotide sequence (SEQ ID NO:9) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:11) of the light (kappa) chain V region of human monoclonal antibody 11B8.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved antibody-based therapies for treating and diagnosing a variety of disorders involving cells expressing CD20. Therapies of the invention employ isolated human monoclonal antibodies which

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specifically bind to an epitope present on CD20. Isolated human monoclonal antibodies encompassed by the present invention include IgA, IgG1-4, IgE, IgM, and IgD antibodies.

In one embodiment the antibody is an IgG1 antibody, more particularly an IgG1,κ or IgG1,λ isotype. In another embodiment the antibody is an IgG3 antibody, more particularly an IgG3,κ or IgG3,λ isotype. In yet another embodiment the antibody is an IgG4 antibody, more particularly an IgG4,κ or IgG4,λ isotype. In still another embodiment the antibody is an IgA1 or IgA2 antibody.

In still another embodiment the antibody is an IgM antibody.

In one embodiment, the human antibodies are produced in a non-human transgenic animal, *e.g.*, a transgenic mouse, capable of producing multiple isotypes of human monoclonal antibodies to CD20 by undergoing V-D-J recombination and isotype switching. Accordingly, aspects of the invention include not only antibodies, antibody fragments, and pharmaceutical compositions thereof, but also non-human transgenic animals, B cells, host cell transfectomas, and hybridomas which produce monoclonal antibodies. Such transgenic animal can also be a transgenic rabbit for producing polyclonal antibodies such as disclosed in US 2003/0017534. Accordingly, the invention also encompasses human polyclonal antibodies which specifically bind to CD20. In one embodiment the invention relates to polyclonal antibodies which bind to an epitope on CD20 (i) which does not comprise or require the amino acid residue proline at position 172; (ii) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172; (iii) which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; (iv) which does not comprise or require the amino acid residue proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; or (v) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166.

In another embodiment the invention relates to human polyclonal antibodies which have one or more of the following characteristics: (i) bind to mutant P172S CD20 (proline at position 172 mutated to serine) with at least the same affinity as to human CD20; (ii) bind to mutant AxP (alanine at position 170 mutated to serine, and proline at position 172 mutated to serine) with at least the same affinity as to human CD20; (iii) show a reduced binding of 50% or more to mutant N166D (asparagine at position 166 mutated to aspartic acid) to human CD20 at an antibody concentration of 10 μg/ml; and/or (iv) show a reduced binding of 50% or more to mutant N163D (asparagine at position 163 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml.

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In yet another embodiment the invention the invention relates to human polyclonal antibodies which bind to an epitope in the small first extracellular loop of human CD20. In still another embodiment the invention also encompasses human polyclonal antibodies which bind to a discontinuous epitope on CD20. In a further embodiment the invention relates to human polyclonal antibodies which bind a discontinuous epitope on CD20, which has part of the first small extracellular loop and part of the second extracellular loop. In still a further embodiment the invention relates to human polyclonal antibodies which bind to a discontinuous epitope on CD20, which has residues AGIYAP of the small first extracellular loop and residues MESLNFIRAHTPYI of the second extracellular loop.

Methods of using the antibodies of the invention to detect a cell expressing CD20 are encompassed by the invention. Methods of using the antibodies of the invention to block or inhibit CD20 induced activities, *e.g.*, proliferative and/or differentiation activities, are also provided and are useful in the treatment of disorders associated with CD20, such as tumorigenic diseases (*e.g.*, B cell lymphoma) and autoimmune diseases (*e.g.*, RA, Chrohn's disease and Wegener's granulomatosis).

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The terms "CD20" and "CD20 antigen" are used interchangeably herein, and include any variants, isoforms and species homologs of human CD20 which are naturally expressed by cells or are expressed on cells transfected with the CD20 gene. Binding of an antibody of the invention to the CD20 antigen mediate the killing of cells expressing CD20 (*e.g.*, a tumor cell) by inactivating CD20. The killing of the cells expressing CD20 may occur by one or more of the following mechanisms:

complement dependent cytotoxity (CDC) of cells expressing CD20;
apoptosis of cells expressing CD20;
effector cell phagocytosis of cells expressing CD20; or
effector cell antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20.

Synonyms of CD20, as recognized in the art, include B-lymphocyte antigen CD20, B-lymphocyte surface antigen B1, Leu-16, Bp35, BM5, and LF5.

As used herein, the term "inhibits growth" (*e.g.*, referring to cells) is intended to include any measurable decrease in the cell growth when contacted with an anti-CD20 antibody as compared to the growth of the same cells not in contact with an anti-CD20 antibody, *e.g.*, the inhibition of growth of a cell culture by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 99%, or 100%. Such a decrease in cell

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growth can occur by a variety of mechanisms, *e.g.*, effector cell phagocytosis, ADCC, CDC, and/or apoptosis.

The term "raft" refers to the sphingolipid- and cholesterol-rich membrane microdomains located in the outer leaflet area of the plasma membrane of a cell. The ability of certain proteins to associate within such domains can effect the protein's function. For example, the translocation of CD20 molecules into lipid rafts, after being bound by human antibodies of the present invention, creates a high density of CD20 antigen-antibody complexes in the plasma membranes. Such a high density of CD20 antigen-antibody complexes can enable efficient activation of the complement system during CDC.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, CD20). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; (vi) an isolated complementarity determining region (CDR), and (vii) a combination of two or more

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isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883).* Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. A further example is binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. The binding domain polypeptide can be a heavy chain variable region or a light chain variable region. The binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term "discontinuous epitope," as used herein, means a conformational epitope on a protein antigen which is formed from at least two separate regions in the primary sequence of the protein.

The term "bispecific molecule" is intended to include any agent, *e.g.*, a protein, peptide, or protein or peptide complex, which has two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen and (b) an Fc receptor on the surface of an effector cell. The term "multispecific molecule" or "heterospecific molecule" is intended to include any agent, *e.g.*, a protein, peptide, or protein or peptide complex, which has more than two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen, (b) an Fc receptor on the surface of an effector cell, and (c) at least one other component. Accordingly, the invention includes, but is not limited to, bispecific, trispecific, tetraspecific, and other multispecific molecules which are directed to cell

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surface antigens, such as CD20, and to other targets, such as Fc receptors on effector cells.

The term "bispecific antibodies" also includes diabodies. Diabodies are bivalent, bispecific antibodies in which the V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123).

The term "human antibody derivatives" refers to any modified form of the antibody, *e.g.*, a conjugate of the antibody and another agent or antibody.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, *e.g.*, by immunizing a transgenic mouse carrying human immunoglobulin genes or by screening a human immunoglobulin gene library, and wherein the selected human antibody is at least 90%, more preferably at least 95%, even more preferably at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid differences, more preferably, no more than 5, or even more preferably, no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

As used herein, the term "heteroantibodies" refers to two or more antibodies, derivatives therefrom, or antigen binding regions linked together, at least two of which have different specificities. These different specificities include a binding specificity for an Fc receptor on an effector cell, and a binding specificity for an antigen or epitope on a target cell, *e.g.*, a tumor cell.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

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The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further in Section I, below), (b) antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term "transfectoma", as used herein, includes recombinant eukaryotic host cell expressing the antibody, such as CHO cells, NS/0 cells, HEK293 cells, plant cells, or fungi, including yeast cells .

As used herein, a "heterologous antibody" is defined in relation to the transgenic non-human organism producing such an antibody. This term refers to an antibody having an amino acid sequence or an encoding nucleic acid sequence corresponding to that found in an organism not consisting of the transgenic non-human animal, and generally from a species other than that of the transgenic non-human animal.

As used herein, a "heterohybrid antibody" refers to an antibody having a light and heavy chains of different organismal origins. For example, an antibody having a human heavy chain associated with a murine light chain is a heterohybrid antibody.

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Examples of heterohybrid antibodies include chimeric and humanized antibodies, discussed supra.

An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds to CD20 is substantially free of antibodies that specifically bind antigens other than CD20). An isolated antibody that specifically binds to an epitope, isoform or variant of human CD20 may, however, have cross-reactivity to other related antigens, *e.g.*, from other species (*e.g.*, CD20 species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different specificities are combined in a well defined composition.

As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity corresponding to a K_D of about 1 x 10^{-7} M or less, and binds to the predetermined antigen with an affinity corresponding to a K_D that is at least two orders of magnitude lower than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and " an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

As used herein, the term "k_d" (sec^{-1}), as used herein, is intended to refer to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value.

The term "k_a" (M^{-1} x sec^{-1}), as used herein, is intended to refer to the association rate constant of a particular antibody-antigen interaction.

The term "K_D" (M), as used herein, is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction.

The term "K_A" (M^{-1}), as used herein, is intended to refer to the association equilibrium constant of a particular antibody-antigen interaction and is obtained by dividing the k_a by the k_d.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by heavy chain constant region genes.

As used herein, "isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

As used herein, "nonswitched isotype" refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the CH gene encoding the nonswitched isotype is typically the first CH gene immediately

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downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination events which involve at least one switch sequence region in the transgene. Non-classical isotype switching may occur by, for example, homologous recombination between human σ_μ and human $\sum_\mu$ (δ-associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

As used herein, the term "switch sequence" refers to those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, will be 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region will be between the construct region to be deleted and the replacement constant region (*e.g.*, γ, ε, etc.). As there is no specific site where recombination always occurs, the final gene sequence will typically not be predictable from the construct.

As used herein, "glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin (antibody) protein. A glycosylation pattern of a heterologous antibody can be characterized as being substantially similar to glycosylation patterns which occur naturally on antibodies produced by the species of the non-human transgenic animal, when one of ordinary skill in the art would recognize the glycosylation pattern of the heterologous antibody as being more similar to said pattern of glycosylation in the species of the non-human transgenic animal than to the species from which the CH genes of the transgene were derived.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "rearranged" as used herein refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete V_H or V_L domain, respectively. A rearranged immunoglobulin (antibody) gene locus can be identified by comparison to germline DNA; a rearranged locus will have at least one recombined heptamer/nonamer homology element.

The term "unrearranged" or "germline configuration" as used herein in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

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The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding whole antibodies or antibody portions (*e.g.*, V_H, V_L, CDR3) that bind to CD20, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the intact antibody or antibody portion are free of other nucleotide sequences encoding whole antibodies or antibody portions that bind antigens other than CD20, which other sequences may naturally flank the nucleic acid in human genomic DNA. In one embodiment, the human anti-CD20 antibody includes the nucleotide or amino acid sequence of 2F2, 7D8, or 11B8, as well as heavy chain (V_H) and light chain (V_L) variable regions having the sequences shown in SEQ ID NOs: 1, 5, or 9, and SEQ ID NOs: 3, 7, or 11, respectively.

As disclosed and claimed herein, the sequences set forth in SEQ ID NOs: 1-30 include "conservative sequence modifications," *i.e.*, nucleotide and amino acid sequence modifications which do not significantly affect or alter the binding characteristics of the antibody encoded by the nucleotide sequence or containing the amino acid sequence. Such conservative sequence modifications include nucleotide and amino acid substitutions, additions and deletions. Modifications can be introduced into SEQ ID NOs:1-30 by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-CD20 antibody is preferably replaced with another amino acid residue from the same side chain family.

The present invention also encompasses "derivatives" of the amino acid sequences as set forth in SEQ ID NOs: 1-30 and conservative sequence modifications thereof, wherein one or more of the amino acid residues have been derivatised, *e.g.*, by acylation or glycosylation, without significantly affecting or altering the binding characteristics of the antibody containing the amino acid sequences.

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Furthermore, the present invention comprises antibodies in which alterations have been made in the Fc region in order to change the functional or pharmacokinetic properties of the antibodies. Such alterations may result in a decrease or increase of C1q binding and CDC or of FcγR binding and ADCC. Substitutions can, for example, be made in one or more of the amino acid residues at positions 234, 235, 236, 237, 297, 318, 320 and 322 of the heavy chain constant region, thereby causing an alteration in an effector function while retaining the ability to bind to the antigen as compared with the unmodified antibody, cf. US 5,624,821 and US 5,648,260.

The *in vivo* half-life of the antibodies can also be improved by modifying the salvage receptor epitope of the Ig constant domain or an Ig-like constant domain such that the molecule does not comprise an intact CH2 domain or an intact Ig Fc region, cf. US 6,121,022 and US 6,194,551. The *in vivo* half-life can furthermore be increased by making mutations in the Fc region, *e.g.*, by substituting threonine for leucine at position 252, by substituting threonine for serine at position 254, or by substituting threonine for phenylalanine at position 256, cf. US 6,277,375.

Furthermore, the glycosylation pattern of the antibodies can be modified in order to change the effector function of the antibodies. For example, the antibodies can be expressed in a transfectoma which does not add the fucose unit normally attached to Asn at position 297 of the Fc region in order to enhance the affinity of the Fc region for FcγRIII which, in turn, will result in an increased ADCC of the antibodies in the presence of NK cells, cf. Shield *et al.* (2002) *JBC*, 277:26733. Furthermore, modification of galactosylation can be made in order to modify CDC.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a anti-CD20 antibody coding sequence, such as by saturation mutagenesis, and the resulting modified anti-CD20 antibodies can be screened for binding activity.

Accordingly, antibodies encoded by the (heavy and light chain variable region) nucleotide sequences disclosed herein and/or containing the (heavy and light chain variable region) amino acid sequences disclosed herein (*i.e.*, SEQ ID NOs: 1-30) include substantially similar antibodies encoded by or containing similar sequences which have been conservatively modified. Further discussion as to how such substantially similar antibodies can be generated based on the partial (*i.e.*, heavy and light chain variable regions) sequences disclosed herein as SEQ ID Nos:1-30 is provided below.

For nucleic acids, the term "substantial homology" indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about

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98% to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

For nucleotide and amino acid sequences, the term "homology" indicates the degree of identity between two nucleic acid or amino acid sequences when optimally aligned and compared with appropriate insertions or deletions. Alternatively, substantial homology exists when the DNA segments will hybridize under selective hybridization conditions, to the complement of the strand.

The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*,

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(1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See*, F. Ausubel, *et al.*, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures thereof, may be mutated in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription of regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors").

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In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. Recombinant host cells include, for example, transfectomas, such as CHO cells, NS/0 cells, and lymphocytic cells.

As used herein, the term "subject" includes any human or non-human animal. The term "non-human animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

The terms "transgenic, non-human animal" refers to a non-human animal having a genome comprising one or more human heavy and/or light chain transgenes or transchromosomes (either integrated or non-integrated into the animal's natural genomic DNA) and which is capable of expressing fully human antibodies. For example, a transgenic mouse can have a human light chain transgene and either a human heavy chain transgene or human heavy chain transchromosome, such that the mouse produces human anti-CD20 antibodies when immunized with CD20 antigen and/or cells expressing CD20. The human heavy chain transgene can be integrated into the chromosomal DNA of the mouse, as is the case for transgenic, *e.g.*, HuMAb mice, such as HCo7 or HCo12 mice, or the human heavy chain transgene can be maintained extrachromosomally, as is the case for transchromosomal (*e.g.*, KM) mice as described in WO 02/43478. Such transgenic and transchromosomal mice are capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching.

Various aspects of the invention are described in further detail in the following subsections.

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I. Production of Human Antibodies to CD20

Human monoclonal antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed, *e.g.*, viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of human antibody genes.

The preferred animal system for preparing hybridomas that secrete human monoclonal antibodies is the murine system. Hybridoma production in the mouse is a very well established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

In a preferred embodiment, human monoclonal antibodies directed against CD20 can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as HuMAb mice and KM mice, respectively, and are collectively referred to herein as "transgenic mice."

The HuMAb mouse contains a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. *et al.* (1994) *Nature* 368 (6474): 856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ and in response to immunization, the introduced human heavy and light chain transgenes, undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal antibodies (Lonberg, N. *et al.* (1994), supra; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764: 536-546). The preparation of HuMAb mice is described in detail in Taylor, L. *et al.* (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. et al (1993) *International Immunology* 5: 647-656; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912-2920; Lonberg *et al.*, (1994) *Nature* 368(6474): 856-859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylor, L. *et al.* (1994) *International Immunology* 6: 579-591; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13:65-93; Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546; Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14:845-851. See further, US Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay, as well as US 5,545,807 to Surani *et al.*; WO

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98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918 and WO 01/09187.

The KM mouse contains a human heavy chain transchromosome and a human kappa light chain transgene. The endogenous mouse heavy and light chain genes also have been disrupted in the KM mice such that immunization of the mice leads to production of human immunoglobulins rather than mouse immunoglobulins. Construction of KM mice and their use to raise human immunoglobulins is described in detail in WO 02/43478.

Immunizations

To generate fully human monoclonal antibodies to CD20, transgenic or transchromosomal mice containing human immunoglobulin genes (*e.g.*, HCo12, HCo7 or KM mice) can be immunized with an enriched preparation of CD20 antigen and/or cells expressing CD20, as described, for example, by Lonberg *et al.* (1994), supra; Fishwild *et al.* (1996), *supra*, and WO 98/24884. Alternatively, mice can be immunized with DNA encoding human CD20. Preferably, the mice will be 6-16 weeks of age upon the first infusion. For example, an enriched preparation (5-50 μg) of the CD20 antigen can be used to immunize the HuMAb mice intraperitoneally. In the event that immunizations using a purified or enriched preparation of the CD20 antigen do not result in antibodies, mice can also be immunized with cells expressing CD20, *e.g.*, a cell line, to promote immune responses.

Cumulative experience with various antigens has shown that the HuMAb transgenic mice respond best when initially immunized intraperitoneally (IP) or subcutaneously (SC) with CD20 expressing cells in complete Freund's adjuvant, followed by every other week IP immunizations (up to a total of 10) with CD20 expressing cells in PBS. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by FACS analysis (as described below), and mice with sufficient titers of anti-CD20 human immunoglobulin can be used for fusions. Mice can be boosted intravenously with CD20 expressing cells 3 days before sacrifice and removal of the spleen.

Generation of Hybridomas Producing Human Monoclonal Antibodies to CD20

To generate hybridomas producing human monoclonal antibodies to human CD20, splenocytes and lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can then be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from

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immunized mice can be fused to SP2/0-Ag8.653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50% PEG (w/v). Cells can be plated at approximately 1 x 10^5 per well in flat bottom microtiter plate, followed by a two week incubation in selective medium containing besides usual reagents 10% fetal Clone Serum, 5-10% origen hybridoma cloning factor (IGEN) and 1X HAT (Sigma). After approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human kappa-light chain containing antibodies and by FACS analysis using CD20 expressing cells for CD20 specificity. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again, and if still positive for human IgG, anti-CD20 monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured *in vitro* to generate antibody in tissue culture medium for characterization.

Generation of Transfectomas Producing Human Monoclonal Antibodies to CD20

Human antibodies of the invention also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art (Morrison, S. (1985) Science 229:1202).

For example, in one embodiment, the gene(s) of interest, *e.g.*, human antibody genes, can be ligated into an expression vector such as a eukaryotic expression plasmid such as used by the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338 841 or other expression systems well known in the art. The purified plasmid with the cloned antibody genes can be introduced in eukaryotic host cells such as CHO cells, NS/0 cells or HEK293 cells or alternatively other eukaryotic cells like a plant derived cells, fungi or yeast cells. The method used to introduce these genes could be methods described in the art such as electroporation, lipofectine, lipofectamine or other. After introducing these antibody genes in the host cells, cells expressing the antibody can be identified and selected. These cells represent the transfectomas which can then be amplified for their expression level and upscaled to produce antibodies. Recombinant antibodies can be isolated and purified from these culture supernatants and/or cells.

Further Recombinant Means for Producing Human Monoclonal Antibodies to CD20

Alternatively, the cloned antibody genes can be expressed in other expression systems, including prokaryotic cells, such as microorganisms, such as *E. coli*, for the production of single chain Fv antibodies, algi, as well as insect cells. Furthermore, the antibodies can be produced in transgenic non-human animals, such as

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in milk from sheep and rabbits or in eggs from hens, or in transgenic plants. See *e.g.* Verma, R., *et al.* (1998). Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression systems. *J.Immunol.Meth.* 216:165-181; Pollock, *et al.* (1999). Transgenic milk as a method for the production of recombinant antibodies. *J.Immunol.Meth.* 231:147-157; and Fischer, R., *et al.* (1999). Molecular farming of recombinant antibodies in plants. *Biol.Chem.* 380:825-839.

Use of Partial Antibody Sequences to Express Intact Antibodies

Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.* (1998) *Nature* 332:323-327; Jones, P. *et al.* (1986) *Nature* 321:522-525; and Queen, C. *et al.* (1989) *Proc. Natl. Acad. Sci.*. U.S.A. 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequences of a high affinity secondary repertoire antibody at individual evenly across the variable region. For example, somatic mutations are relatively infrequent in the amino terminal portion of framework region 1 and in the carboxy-terminal portion of framework region 4. Furthermore, many somatic mutations do not significantly alter the binding properties of the antibody. For this reason, it is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody (see WO 99/45962). Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in missing portions of the variable regions. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire variable

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region can be synthesized as a set of short, overlapping, oligonucleotides and combined by PCR amplification to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion or particular restriction sites, or optimization of particular codons.

The nucleotide sequences of heavy and light chain transcripts from hybridomas are used to design an overlapping set of synthetic oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and kappa chain sequences can differ from the natural sequences in three ways: strings of repeated nucleotide bases are interrupted to facilitate oligonucleotide synthesis and PCR amplification; optimal translation initiation sites are incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266:19867-19870); and HindIII sites are engineered upstream of the translation initiation sites.

For both the heavy and light chain variable regions, the optimized coding and corresponding non-coding, strand sequences are broken down into 30 - 50 nucleotides approximately at the midpoint of the corresponding non-coding oligonucleotide. Thus, for each chain, the oligonucleotides can be assembled into overlapping double stranded sets that span segments of 150 - 400 nucleotides. The pools are then used as templates to produce PCR amplification products of 150 - 400 nucleotides. Typically, a single variable region oligonucleotide set will be broken down into two pools which are separately amplified to generate two overlapping PCR products. These overlapping products are then combined by PCR amplification to form the complete variable region. It may also be desirable to include an overlapping fragment of the heavy or light chain constant region (including the BbsI site of the kappa light chain, or the AgeI site if the gamma heavy chain) in the PCR amplification to generate fragments that can easily be cloned into the expression vector constructs.

The reconstructed heavy and light chain variable regions are then combined with cloned promoter, leader sequence, translation initiation, constant region, 3' untranslated, polyadenylation, and transcription termination, sequences to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a host cell expressing both chains.

Plasmids for use in construction of expression vectors for human IgGκ are described below. The plasmids were constructed so that PCR amplified V heavy and V kappa light chain cDNA sequences could be used to reconstruct complete heavy and light chain minigenes. These plasmids can be used to express completely human, or chimeric IgG1,κ or IgG4,κ antibodies. Similar plasmids can be constructed for

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expression of other heavy chain isotypes, or for expression of antibodies comprising lambda light chains.

Thus, in another aspect of the invention, the structural features of the human anti-CD20 antibodies of the invention, *e.g.*, 11B8, 2F2, or 7D8, are used to create structurally related human anti-CD20 antibodies that retain at least one functional property of the antibodies of the invention, such as binding to CD20. More specifically, one or more CDR regions of 2F2, 7D8, or 11B8 can be combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, human anti-CD20 antibodies of the invention.

Accordingly, in another embodiment, the invention provides a method for preparing an anti-CD20 antibody comprising:

preparing an antibody comprising (1) human heavy chain framework regions and human heavy chain CDRs, wherein at least one of the human heavy chain CDRs comprises an amino acid sequence selected from the amino acid sequences of CDRs shown in Figures 53, 55, or 57 (or corresponding amino acid residues in SEQ ID NOs:13-15, 19-21, or 25-27); and (2) human light chain framework regions and human light chain CDRs, wherein at least one of the human light chain CDRs comprises an amino acid sequence selected from the amino acid sequences of CDRs shown in Figures 53, 55, or 57 (or corresponding amino acid residues in SEQ ID NOs: 16-18, 22-24, or 28-30); wherein the antibody retains the ability to bind to CD20.

The ability of the antibody to bind CD20 can be determined using standard binding assays, such as those set forth in the Examples (*e.g.*, a FACS analysis).

Since it is well known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen, the recombinant antibodies of the invention prepared as set forth above preferably comprise the heavy and light chain CDR3s of 2F2, 7D8, or 11B8. The antibodies further can comprise the CDR2s of 2F2, 7D8, or 11B8. The antibodies further can comprise the CDR1s of 2F2, 7D8, or 11B8. Accordingly, the invention further provides anti-CD20 antibodies comprising: (1) human heavy chain framework regions, a human heavy chain CDR1 region, a human heavy chain CDR2 region, and a human heavy chain CDR3 region, wherein the human heavy chain CDR3 region is the CDR3 of 2F2, 7D8, or 11B8 as shown in Figures 53, 55, or 57 (or corresponding amino acid residues as shown in SEQ ID NOs: 15, 21, or 27); and (2) human light chain framework regions, a human light chain CDR1 region, a human light chain CDR2 region, and a human light chain CDR3 region, wherein the human light chain CDR3 region is the CDR3 of 2F2, 7D8, or 11B8 as shown in Figures 53, 55, or 57 (or corresponding amino acid residues as shown in SEQ ID NOs:18, 24, or 30), wherein the antibody binds CD20. The antibody may further comprise the heavy chain CDR2 and/or

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the light chain CDR2 of 2F2, 7D8, or 11B8. The antibody may further comprise the heavy chain CDR1 and/or the light chain CDR1 of 2F2, 7D8, or 11B8.

Preferably, the CDR1, 2, and/or 3 of the engineered antibodies described above comprise the exact amino acid sequence(s) as those of 2F2, 7D8, or 11B8 disclosed herein. However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences of 2F2, 7D8, or 11B8 may be possible while still retaining the ability of the antibody to bind CD20 effectively (*e.g.*, conservative substitutions). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 90%, 95%, 98% or 99.5% identical to one or more CDRs of 2F2, 7D8, or 11B8.

In addition to simply binding CD20, engineered antibodies such as those described above may be selected for their retention of other functional properties of antibodies of the invention, such as:

(1) low dissociation rate from CD20;

(2) high affinity binding to CD20;

(3) binding to a unique epitope on CD20, and/or binding in a specific orientation to CD20, and/or binding to a specific form of CD20;

(4) mediation of a high level of CDC on either CD55/59 negative or CD55/59 positive cells;

(5) translocation into lipid rafts upon binding to CD20;

(6) inhibition of the growth of cells which express CD20;

(7) inducement of apoptosis of cells which express CD20;

(8) inducement of homotypic adhesion of cells which express CD20;

(9) prolonged survival of a subject having tumor cells which express CD20;

(10) mediation of ADCC of CD20 targets when mixed with appropriate effector cells;

(11) ability to deplete cells which express CD20; and/or

(12) ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

Characterization of Binding of Human Monoclonal Antibodies to CD20

To purify human anti-CD20 antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (for IgG1 isotype antibodies) (Pharmacia, Piscataway, NJ) or anti-human IgG coated sepharose or protein G-sepharose in case of IgG3 isotype antibodies. Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to

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ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD_{280} using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80 °C.

To determine if the selected human anti-CD20 monoclonal antibodies bind to unique epitopes, site-directed or multi-site directed mutagenesis can be used.

To determine the isotype of purified antibodies, isotype ELISAs can be performed. Wells of microtiter plates can be coated with 10 μg/ml of anti-human Ig overnight at 4 °C. After blocking with 5% BSA, the plates are reacted with 10 μg/ml of monoclonal antibodies or purified isotype controls, at ambient temperature for two hours. The wells can then be reacted with either human IgGl, IgG2, IgG3 or IgG4 or human IgM-specific alkaline phosphatase-conjugated probes. After washing, the plates are developed with pNPP substrate (1 mg/ml) and analyzed at OD of 405-650.

In order to demonstrate presence of anti-CD20 antibodies in sera of immunized mice or binding of monoclonal antibodies to live cells expressing the CD20, flow cytometry can be used. Briefly, cell lines expressing CD20 (grown under standard growth conditions) are mixed with various concentrations of monoclonal antibodies in PBS containing 0.1% BSA and 0.02% sodium-azide, and incubated at 4 °C for 30 min. After washing, the cells are reacted with fluorescein-labeled anti-human IgG antibody under the same conditions as the primary antibody staining. The samples can be analyzed by flow cytometry with a FACS instrument using light and side scatter properties to gate on single, living cells. An alternative assay using fluorescence microscopy may be used (in addition to or instead of) the flow cytometry assay. Cells can be stained exactly as described above and examined by fluorescence microscopy. This method allows visualization of individual cells, but may have diminished sensitivity depending on the density of the antigen.

Anti-CD20 human IgGs can be further tested for reactivity with CD20 antigen by Western blotting. Briefly, cell extracts from cells expressing CD20 can be prepared and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens will be transferred to nitrocellulose membranes, blocked with 20% mouse serum, and probed with the monoclonal antibodies to be tested. Human IgG binding can be detected using anti-human IgG alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, MO).

Phagocytic and Cell Killing Activities of Human Monoclonal Antibodies to CD20

In addition to binding specifically to CD20, human monoclonal anti-CD20 antibodies can be tested for their ability to mediate phagocytosis and killing of cells expressing CD20. The testing of monoclonal antibody activity *in vitro* will provide

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an initial screening prior to testing *in vivo* models. Briefly, polymorphonuclear cells (PMNs), NK cells, monocytes or other effector cells, from healthy donors can be purified by Ficoll Hypaque density centrifugation, followed by lysis of contaminating erythrocytes. Washed PMNs, can be suspended in RPMI supplemented with 10% heat-inactivated fetal calf serum and mixed with ^{51}Cr labeled cells expressing CD20, at various ratios of effector cells to tumor cells (-effector cells:tumor cells). Purified human anti-CD20 IgGs can then be added at various concentrations. Irrelevant human IgG can be used as negative control. Assays can be carried out for 4 to 20 hours at 37 °C depending on the effector cell type used. Samples can be assayed for cytolysis by measuring ^{51}Cr release into the culture supernatant. Anti-CD20 monoclonal antibodies can also be tested in combinations with each other to determine whether cytolysis is enhanced with multiple monoclonal antibodies.

Human monoclonal antibodies which bind to CD20 also can be tested in an *in vivo* model (*e.g.*, in mice) to determine their efficacy in controlling growth of CD20-expressing tumor cells. These antibodies can be selected, for example, based on the following criteria, which are not intended to be exclusive:

1. binding to live cells expressing CD20;
2. low dissociation rate from CD20;
3. high affinity of binding to CD20;
4. binding to a unique epitope on CD20; and/or binding in a specific orientation to CD20, and/or binding to a specific form of CD20;
5. opsonization of cells expressing CD20;
6. mediation of growth inhibition, phagocytosis and/or killing of cells expressing CD20 in the presence of human effector cells;
7. ability to induce CDC on either CD55/CD59 negative or positive cells;
8. ability to induce homotypic adhesion;
9. ability to induce translocation into lipid rafts upon binding to CD20;
10. ability to induce apoptosis;
11. ability to induce ADCC on cells expressing CD20;
12. ability to deplete cells which express CD20; and/or
13. ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

Preferred human monoclonal antibodies of the invention meet one or more of these criteria.

Human monoclonal anti-CD20 antibodies can be tested for their ability to mediate CDC using a variety of known techniques. For example, serum for complement can be obtained from the blood of healthy subjects which can be centrifuged and harvested. To determine the CDC activity of various mAbs, different methods can be used. ^{51}Cr release can for example be measured or elevated membrane permeability can

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be assessed using a propidium iodide (PI) exclusion assay. Briefly, target cells can be washed and resuspended in RPMI-1% BSA at 1 x 10^6/ml. Various concentrations of mAb can be added to the cells and allowed to bind for 10-15 min at room temperature. Serum can then be added to a final concentration of 20% (v/v) and the cells incubated at 37 °C for 45 min. All cells from each sample can be added to the PI solution in a FACS tube. The mixture can then be assessed immediately by flow cytometry using a FACScalibur flow cytometer and analysed using CellQuest pro software (BD Biosciences, Mountain view, CA).

To test for the ability to initiate apoptosis, human monoclonal anti-CD20 antibodies can, for example, be incubated with CD20 positive tumor cells, *e.g.*, Daudi at 37 °C for about 20 hours. The cells can be harvested, washed in Annexin-V-FITC binding buffer (BD biosciences), and labeled with Annexin V-FITC (BD biosciences) for 15 min in the dark at 4 °C. All cells from each sample can be added to PI solution (10 μg/ml in PBS) in a FACS tube and assessed immediately by flow cytometry (as above).

In a particular embodiment of the invention, the human monoclonal antibodies are used in combination, *e.g.*, as a pharmaceutical composition comprising two or more anti-CD20 monoclonal antibodies. For example, human anti-CD20 monoclonal antibodies having different but complementary activities can be combined in a single therapy to achieve a desired therapeutic or diagnostic effect. In a preferred embodiment, the composition includes an anti-CD20 human monoclonal antibody that mediates CDC combined with another human anti-CD20 monoclonal antibody that induces apoptosis. In another embodiment, the composition includes an anti-CD20 human monoclonal antibody that mediates highly effective killing of target cells in the presence of effector cells, combined with another human anti-CD20 monoclonal antibody that inhibits the growth of cells expressing CD20.

II. Production of Transgenic Non-human Animals Which Generate Human Monoclonal Anti-CD20 Antibodies

In yet another aspect, the invention provides transgenic and transchromosomal non-human animals, such as transgenic or transchromosomal mice, which are capable of expressing human antibodies that specifically bind to CD20. In a particular embodiment, the invention provides a transgenic or transchromosomal mouse having a genome comprising a human heavy chain transgene, such that the mouse produces human anti- CD20 antibodies when immunized with cells expressing CD20. The human heavy chain transgene can be integrated into the chromosomal DNA of the mouse, as is the case for transgenic, *e.g.*, HuMAb mice, as described in detail herein and exemplified. Alternatively, the human heavy chain transgene can be maintained

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extrachromosomally, as is the case for transchromosomal (*e.g.*, KM) mice as described in WO 02/43478. Such transgenic and transchromosomal animals are capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J/V-J recombination and isotype switching. The design of a transgenic or transchromosomal non-human animal that responds to foreign antigen stimulation with a heterologous antibody repertoire, requires that the heterologous immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B cell development. This includes, for example, isotype switching of the heterologous heavy chain transgene. Accordingly, transgenes are constructed so as that isotype switching can be induced and one or more of the following characteristics of antibody genes: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

Not all of the foregoing criteria need be met. For example, in those embodiments wherein the endogenous immunoglobulin loci of the transgenic animal are functionally disrupted, the transgene need not activate allelic exclusion. Further, in those embodiments wherein the transgene comprises a functionally rearranged heavy and/or light chain immunoglobulin gene, the second criteria of functional gene rearrangement is unnecessary, at least for that transgene which is already rearranged. For background on molecular immunology, see, Fundamental Immunology, 2nd edition (1989), Paul William E., ed. Raven Press, N.Y.

In certain embodiments, the transgenic or transchromosomal non-human animals used to generate the human monoclonal antibodies of the invention contain rearranged, unrearranged or a combination of rearranged and unrearranged heterologous immunoglobulin heavy and light chain transgenes in the germline of the transgenic animal. Each of the heavy chain transgenes comprises at least one C_H gene. In addition, the heavy chain transgene may contain functional isotype switch sequences, which are capable of supporting isotype switching of a heterologous transgene encoding multiple C_H genes in the B cells of the transgenic animal. Such switch sequences may be those which occur naturally in the germline immunoglobulin locus from the species that serves as the source of the transgene C_H genes, or such switch sequences may be derived from those which occur in the species that is to receive the transgene construct (the transgenic animal). For example, a human transgene construct that is used to produce a transgenic mouse may produce a higher frequency of isotype switching events if it incorporates switch sequences similar to those that occur naturally in the mouse heavy chain locus, as presumably the mouse switch sequences are optimized to function with the mouse

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switch recombinase enzyme system, whereas the human switch sequences are not. Switch sequences may be isolated and cloned by conventional cloning methods, or may be synthesized *de novo* from overlapping synthetic oligonucleotides designed on the basis of published sequence information relating to immunoglobulin switch region sequences (Mills *et al.*, *Nucl. Acids Res.* 15:7305-7316 (1991); Sideras *et al.*, *Intl. Immunol.* 1:631-642 (1989)). For each of the foregoing transgenic animals, functionally rearranged heterologous heavy and light chain immunoglobulin transgenes are found in a significant fraction of the B cells of the transgenic animal (at least 10 percent).

The transgenes used to generate the transgenic non-human animals of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and at least one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of the transgenic non-human animal. In one aspect of the invention, the transgene is constructed such that the individual gene segments are unrearranged, *i.e.*, not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes support recombination of the V, D, and J gene segments (functional rearrangement) and preferably support incorporation of all or a portion of a D region gene segment in the resultant rearranged immunoglobulin heavy chain within the transgenic animal when exposed to CD20 antigen.

In an alternate embodiment, the transgenes comprise an unrearranged "mini-locus". Such transgenes typically comprise a substantial portion of the C, D, and J segments as well as a subset of the V gene segments. In such transgene constructs, the various regulatory sequences, *e.g.* promoters, enhancers, class switch regions, splice-donor and splice-acceptor sequences for RNA processing, recombination signals and the like, comprise corresponding sequences derived from the heterologous DNA. Such regulatory sequences may be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments may be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse. Alternatively, synthetic regulatory sequences may be incorporated into the transgene, wherein such synthetic regulatory sequences are not homologous to a functional DNA sequence that is known to occur naturally in the genomes of mammals. Synthetic regulatory sequences are designed according to consensus rules, such as, for example, those specifying the

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permissible sequences of a splice-acceptor site or a promoter/enhancer motif. For example, a minilocus comprises a portion of the genomic immunoglobulin locus having at least one internal (*i.e.*, not at a terminus of the portion) deletion of a non-essential DNA portion (*e.g.*, intervening sequence; intron or portion thereof) as compared to the naturally-occurring germline Ig locus.

Preferred transgenic and transchromosomal non-human animals, *e.g.*, mice, will exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a human after adjusting for volume.

The repertoire will ideally approximate that shown in a human when adjusted for volume, usually with a diversity at least about 10% as great, preferably 25 to 50% or more. Generally, at least about a thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending on the number of different V, J and D regions introduced into the mouse genome and driven by the additional diversity generated by V(-D-)J gene segment rearrangements random nucleotide additions at the joining regions. Typically, the immunoglobulins will exhibit an affinity (K_D) for preselected antigens of below 10^{-7} M, such as of below 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower.

Transgenic and transchromosomal non-human animals, *e.g.*, mice, as described above can be immunized with, for example, cells expressing CD20. Alternatively, the transgenic animals can be immunized with DNA encoding human CD20. The animals will then produce B cells which undergo class-switching via switch recombination (cis-switching) and express immunoglobulins reactive with CD20. The immunoglobulins can be human antibodies (also referred to as "human sequence antibodies"), wherein the heavy and light chain polypeptides are encoded by human transgene sequences, which may include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences; these human antibodies can be referred to as being substantially identical to a polypeptide sequence encoded by a human V_L and J_L or V_H, D_H and J_H gene segments, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. The variable regions of each antibody chain are typically at least 80 percent similar to human germline V, J, and, in the case of heavy chains, D, gene segments; frequently at least 85 percent similar to human germline sequences present on the transgene; often 90 or 95 percent or more similar to human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic mutation and VJ and VDJ joining, the human sequence antibodies will frequently have some variable region sequences which are not encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide

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positions) will cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

Another aspect of the invention includes B cells derived from transgenic or transchromosomal non-human animals as described herein. The B cells can be used to generate hybridomas expressing human monoclonal antibodies which bind with high affinity (*e.g.*, a dissociation equilibrium constant (K_D) of lower than 10^{-7} M) to human CD20. Thus, in another embodiment, the invention provides a hybridoma which produces a human antibody having an affinity (K_D) of below 10^{-7} M, such as of below 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by scatchard analysis of CD20 expressing cells using a radio-actively labeled monoclonal antibody or by determination of the half-maximal binding concentration using FACS analysis.

Herein the monoclonal antibody comprises a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, a D region, and a human J_H segment, and (2) a constant region encoded by a human C_H gene segment.

The development of high affinity human monoclonal antibodies against CD20 can be facilitated by a method for expanding the repertoire of human variable region gene segments in a transgenic non-human animal having a genome comprising an integrated human immunoglobulin transgene, said method comprising introducing into the genome a V gene transgene comprising V region gene segments which are not present in said integrated human immunoglobulin transgene. Often, the V region transgene is a yeast artificial chromosome comprising a portion of a human V_H or V_L (V_K) gene segment array, as may naturally occur in a human genome or as may be spliced together separately by recombinant methods, which may include out-of-order or omitted V gene segments. Often at least five or more functional V gene segments are contained on the YAC. In this variation, it is possible to make a transgenic animal produced by the V repertoire expansion method, wherein the animal expresses an immunoglobulin chain comprising a variable region sequence encoded by a V region gene segment present on the V region transgene and a C region encoded on the human Ig transgene. By means of the V repertoire expansion method, transgenic animals having at least 5 distinct V genes can be generated; as can animals containing at least about 24 V genes or more. Some V gene segments may be non-functional (*e.g.*,

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pseudogenes and the like); these segments may be retained or may be selectively deleted by recombinant methods available to the skilled artisan, if desired.

Once the mouse germline has been engineered to contain a functional YAC having an expanded V segment repertoire, substantially not present in the human Ig transgene containing the J and C gene segments, the trait can be propagated and bred into other genetic backgrounds, including backgrounds where the functional YAC having an expanded V segment repertoire is bred into a non-human animal germline having a different human Ig transgene. Multiple functional YACs having an expanded V segment repertoire may be bred into a germline to work with a human Ig transgene (or multiple human Ig transgenes). Although referred to herein as YAC transgenes, such transgenes when integrated into the genome may substantially lack yeast sequences, such as sequences required for autonomous replication in yeast; such sequences may optionally be removed by genetic engineering (*e.g.*, restriction digestion and pulsed-field gel electrophoresis or other suitable method) after replication in yeast is no longer necessary (*i.e.*, prior to introduction into a mouse ES cell or mouse prozygote). Methods of propagating the trait of human sequence immunoglobulin expression, include breeding a transgenic animal having the human Ig transgene(s), and optionally also having a functional YAC having an expanded V segment repertoire. Both V_H and V_L gene segments may be present on the YAC. The transgenic animal may be bred into any background desired by the practitioner, including backgrounds harboring other human transgenes, including human Ig transgenes and/or transgenes encoding other human lymphocyte proteins. The invention also provides a high affinity human sequence immunoglobulin produced by a transgenic mouse having an expanded V region repertoire YAC transgene. Although the foregoing describes a preferred embodiment of the transgenic animal of the invention, other embodiments are contemplated which have been classified in three categories:

I. Transgenic animals containing an unrearranged heavy and rearranged light chain immunoglobulin transgene;

II. Transgenic animals containing an unrearranged heavy and unrearranged light chain immunoglobulin transgene; and

III. Transgenic animal containing rearranged heavy and an unrearranged light chain immunoglobulin transgene.

Of these categories of transgenic animal, the preferred order of preference is as follows II > I > III where the endogenous light chain genes (or at least the K gene) have been knocked out by homologous recombination (or other method) and I > II > III where the endogenous light chain genes have not been knocked out and must be dominated by allelic exclusion.

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III. Bispecific/ Multispecific Molecules Which Bind to CD20

In yet another embodiment of the invention, human monoclonal antibodies to CD20 can be derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, an Fab' fragment) to generate a bispecific or multispecific molecule which binds to multiple binding sites or target epitopes. For example, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, peptide or binding mimetic.

Accordingly, the present invention includes bispecific and multispecific molecules comprising at least one first binding specificity for CD20 and a second binding specificity for a second target epitope. In a particular embodiment of the invention, the second target epitope is an Fc receptor, *e.g.*, human FcγRI (CD64) or a human Fcα receptor (CD89), or a T cell receptor, *e.g.*, CD3. Therefore, the invention includes bispecific and multispecific molecules capable of binding both to FcγR, FcαR or FcεR expressing effector cells (*e.g.*, monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing CD20. These bispecific and multispecific molecules target CD20 expressing cells to effector cell and, like the human monoclonal antibodies of the invention, trigger Fc receptor-mediated effector cell activities, such as phagocytosis of a CD20 expressing cells, antibody dependent cellular cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

Bispecific and multispecific molecules of the invention can further include a third binding specificity, in addition to an anti-Fc binding specificity and an anti-CD20 binding specificity. In one embodiment, the third binding specificity is an anti-enhancement factor (EF) portion, *e.g.*, a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, *e.g.*, an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the F_c receptor or target cell antigen. The "anti-enhancement factor portion" can bind an F_c receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T cell (*e.g.*, via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell that results in an increased immune response against the target cell).

In one embodiment, the bispecific and multispecific molecules of the invention comprise as a binding specificity at least one antibody, including, *e.g.*, an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as

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described in Ladner *et al.* US 4,946,778. The antibody may also be a binding-domain immunoglobulin fusion protein as disclosed in US 2003/0118592 and US 2003/0133939.

In one embodiment bispecific and multispecific molecules of the invention comprise a binding specificity for an FcγR or an FcαR present on the surface of an effector cell, and a second binding specificity for a target cell antigen, *e.g.*, CD20.

In one embodiment, the binding specificity for an Fc receptor is provided by a human monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ-chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), FcγRII(CD32), and FcγRIII (CD16). In one preferred embodiment, the Fcγ receptor is a human high affinity FcγRI.

The production and characterization of these preferred monoclonal antibodies are described by Fanger *et al.* in WO 88/00052 and in US 4,954,617. These antibodies bind to an epitope of FcγRI, FcγRII or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. In other embodiments, the anti-Fcγ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and characterization of the H22 antibody is described in Graziano, R.F. *et al.* (1995) J. Immunol 155 (10): 4996-5002 and WO 94/10332. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession No. CRL 11177.

In still other preferred embodiments, the binding specificity for an Fc receptor is provided by an antibody that binds to a human IgA receptor, *e.g.*, an Fc-alpha receptor (FcαRI (CD89)), the binding of which is preferably not blocked by human immunoglobulin A (IgA). The term "IgA receptor" is intended to include the gene product of one α-gene (FcαRI) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcαRI (CD89) is constitutively expressed on monocytes/macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαRI has medium affinity for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton, H.C. *et al.* (1996) Critical Reviews in Immunology 16:423-440). Four FcαRI-specific monoclonal antibodies, identified as A3, A59, A62 and A77, which bind FcαRI outside the IgA ligand binding domain, have been described (Monteiro, R.C. *et al.* (1992) *J. Immunol.* 148:1764).

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FcαRI and FcγRI are preferred trigger receptors for use in the invention because they are (1) expressed primarily on immune effector cells, *e.g.*, monocytes, PMNs, macrophages and dendritic cells; (2) expressed at high levels (*e.g.*, 5,000-100,000 per cell); (3) mediators of cytotoxic activities (*e.g.*, ADCC, phagocytosis); (4) mediate enhanced antigen presentation of antigens, including self-antigens, targeted to them.

In another embodiment the bispecific molecule is comprised by two human monoclonal antibodies according to the invention which have complementary functional activities, such as one antibody predominately working by inducing CDC and the other antibody predominately working by inducing apoptosis, *e.g.*, 2F2 in combination with 11B8.

In other embodiments, bispecific and multispecific molecules of the invention further comprise a binding specificity which recognizes, *e.g.*, binds to, a target cell antigen, *e.g.*, CD20. In a preferred embodiment, the binding specificity is provided by a human monoclonal antibody of the present invention.

An "effector cell specific antibody" as used herein refers to an antibody or functional antibody fragment that binds the Fc receptor of effector cells. Preferred antibodies for use in the subject invention bind the Fc receptor of effector cells at a site which is not bound by endogenous immunoglobulin.

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, *e.g.*, lymphocytes (*e.g.*, B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and basophils. Some effector cells express specific Fc receptors and carry out specific immune functions. In preferred embodiments, an effector cell is capable of inducing antibody-dependent cellular cytotoxicity (ADCC), *e.g.*, a neutrophil capable of inducing ADCC. For example, monocytes, macrophages, which express FcR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens. In other embodiments, an effector cell can phagocytose a target antigen, target cell, or microorganism. The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon gamma (IFN-γ). This enhanced expression increases the cytotoxic activity of FcγRI-bearing cells against targets. An effector cell can phagocytose or lyse a target antigen or a target cell.

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"Target cell" shall mean any undesirable cell in a subject (*e.g.*, a human or animal) that can be targeted by a composition (*e.g.*, a human monoclonal antibody, a bispecific or a multispecific molecule) of the invention. In preferred embodiments, the target cell is a cell expressing or overexpressing CD20. Cells expressing CD20 typically include B cells and B cell tumors.

While human monoclonal antibodies are preferred, other antibodies which can be employed in the bispecific or multispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies. Such murine, chimeric and humanized monoclonal antibodies can be prepared by methods known in the art.

Bispecific and multispecific molecules of the present invention can be made using chemical techniques (see *e.g.*, D. M. Kranz *et al.* (1981) *Proc. Natl. Acad. Sci.* USA 78:5807), "polydoma" techniques (See US 4,474,893, to Reading), or recombinant DNA techniques.

In particular, bispecific and multispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, *e.g.*, the anti-FcR and anti-CD20 binding specificities, using methods known in the art and described in the examples provided herein. For example, each binding specificity of the bispecific and multispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (see *e.g.*, Karpovsky *et al.* (1984) *J. Exp. Med.* 160:1686; Liu, MA *et al.* (1985) *Proc. Natl. Acad. Sci.* USA 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan *et al.* (*Science* (1985) 229:81-83), and Glennie *et al.* (*J. Immunol.* (1987) 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific and multispecific molecule is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A bispecific and multispecific molecule of the invention, *e.g.*, a bispecific molecule can be a single chain molecule, such as a single

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chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific and multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- and multispecific molecules are described for example in US 5,260,203; US 5,455,030; US 4,881,175; US 5,132,405; US 5,091,513; US 5,476,786; US 5,013,653; US 5,258,498; and US 5,482,858.

Binding of the bispecific and multispecific molecules to their specific targets can be confirmed by enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), FACS analysis, a bioassay (*e.g.*, growth inhibition), or a Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using *e.g.*, an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

IV. Immunoconjugates

In another aspect, the present invention features a human anti-CD20 monoclonal antibody conjugated to a therapeutic moiety, such as a cytotoxin, a drug (*e.g.*, an immunosuppressant) or a radioisotope. Such conjugates are referred to herein as "immunoconjugates". Immunoconjugates which include one or more cytotoxins are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (*e.g.*, kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof.

Suitable therapeutic agents for forming immunoconjugates of the invention include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, fludarabin, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine

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(BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine). In a preferred embodiment, the therapeutic agent is a cytotoxic agent or a radiotoxic agent. In another embodiment, the therapeutic agent is an immunosuppressant. In yet another embodiment, the therapeutic agent is GM-CSF. In a preferred embodiment, the therapeutic agent is doxorubicin, cisplatin, bleomycin, sulfate, carmustine, chlorambucil, cyclophosphamide or ricin A.

Antibodies of the present invention also can be conjugated to a radioisotope, *e.g.*, iodine-131, yttrium-90 or indium-111, to generate cytotoxic radiopharmaceuticals for treating a CD20-related disorder, such as a cancer. The antibody conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-γ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

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In a further embodiment, the human monoclonal antibodies according to the invention are attached to a linker-chelator, *e.g.*, tiuxetan, which allows for the antibody to be conjugated to a radioisotope.

V. Pharmaceutical Compositions

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of human monoclonal antibodies of the present invention. The pharmaceutical compositions may be formulated with pharmaceutically acceptable carriers or diluents as well as any other known adjuvants and excipients in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. In one embodiment, the compositions include a combination of multiple (*e.g.*, two or more) isolated human antibodies of the invention which act by different mechanisms, *e.g.*, one antibody which predominately acts by inducing CDC in combination with another antibody which predominately acts by inducing apoptosis.

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-inflammatory agent or at least one immunosuppressive agent. In one embodiment such therapeutic agents include one or more anti-inflammatory agents, such as a steroidal drug or a NSAID (nonsteroidal anti-inflammatory drug). Preferred agents include, for example, aspirin and other salicylates, Cox-2 inhibitors, such as rofecoxib (Vioxx) and celecoxib (Celebrex), NSAIDs such as ibuprofen (Motrin, Advil), fenoprofen (Nalfon), naproxen (Naprosyn), sulindac (Clinoril), diclofenac (Voltaren), piroxicam (Feldene), ketoprofen (Orudis), diflunisal (Dolobid), nabumetone (Relafen), etodolac (Lodine), oxaprozin (Daypro), and indomethacin (Indocin).

In another embodiment, such therapeutic agents include one or more DMARDs, such as methotrexate (Rheumatrex), hydroxychloroquine (Plaquenil), sulfasalazine (Asulfidine), pyrimidine synthesis inhibitors, *e.g.*, leflunomide (Arava), IL-1 receptor blocking agents, *e.g.*, anakinra (Kineret), and TNF-α blocking agents, *e.g.*, etanercept (Enbrel), infliximab (Remicade) and adalimumab.

In another embodiment, such therapeutic agents include one or more immunosuppressive agents, such as cyclosporine (Sandimmune, Neoral) and azathioprine (Imural).

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In yet another embodiment, such therapeutic agents include one or more chemotherapeutics, such as doxorubicin (Adriamycin), cisplatin (Platinol), bleomycin (Blenoxane), carmustine (Gliadel), cyclophosphamide (Cytoxan, Procytox, Neosar), and chlorambucil (Leukeran).

In another embodiment, human antibodies of the present invention may be administered in combination with chlorambucil and prednisolone; cyclophosphamide and prednisolone; cyclophosphamide, vincristine, and prednisone; cyclophosphamide, vincristine, doxorubicin, and prednisone; fludarabine and anthracycline; or in combination with other common multi-drugs regimens for NHL, such as disclosed, *e.g.*, in Non-Hodgkin's Lymphomas: Making sense of Diagnosis, Treatment, and Options, Lorraine Johnston, 1999, O'Reilly and Associates, Inc.

In yet another embodiment, the human antibodies may be administered in conjunction with radiotherapy and/or autologous peripheral stem cell or bone marrow transplantation.

In still another embodiment, the human antibodies may be administered in combination with one or more antibodies selected from anti-CD25 antibodies, anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, anti-CD38 antibodies, anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies (*e.g.*, efalizumab), anti-alpha-4/beta-1 integrin (VLA4) antibodies (*e.g.*, natalizumab), and CTLA4-Ig.

In a particular embodiment, the human monoclonal antibodies are administered in combination with an anti-CD25 antibody for the treatment of bullous pemphigoid, *e.g.*, in patients with graft-versus-host disease.

In another particular embodiment, the human monoclonal antibodies are administered in combination with one or more antibodies selected from anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, and anti-CD38 antibodies for the treatment of malignant diseases.

In still another particular embodiment, the human antibodies are administered in combination with one or more antibodies selected from anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies (*e.g.*, efalizumab), anti-alpha-4/beta-1 integrin (VLA4) antibodies (*e.g* natalizumab), and CTLA4-Ig for the treatment of inflammatory diseases.

In yet a further embodiment, the human antibodies may be administered in combination with an anti-C3b(i) antibody in order to enhance complement activation.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably,

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the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) J. Pharm. Sci. 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for the preparation of such formulations are generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neuroimmunol.* 7:27).

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Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical

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carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

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Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In one embodiment the human monoclonal antibodies of the invention are administered in crystalline form by subcutaneous injection, cf. Yang *et al.* (2003) *PNAS*, 100(12):6934-6939

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (more preferably, 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in

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combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

In one embodiment, the human monoclonal antibodies according to the invention may be administered by infusion in a weekly dosage of 10 to 500 mg/m^2, such as 200 to 400 mg/m^2. Such administration may be repeated, *e.g.*, 1 to 8 times, such as 3 to 5 times. The administration may be performed by continuous infusion over a period of from 2 to 24 hours, such as of from 2 to 12 hours.

In another embodiment, the human monoclonal antibodies are administered by slow continuous infusion over a long period, such as more than 24 hours, in order to reduce toxic side effects.

In still another embodiment the human monoclonal antibodies are administered in a weekly dosage of from 250 mg to 2000 mg, such as for example 300 mg, 500 mg, 700 mg, 1000 mg, 1500 mg or 2000 mg, for up to 8 times, such as from 4 to 6 times. The administration may be performed by continuous infusion over a period of from 2 to 24 hours, such as of from 2 to 12 hours. Such regimen may be repeated one or more times as necessary, for example, after 6 months or 12 months. The dosage can be determined or adjusted by measuring the amount of circulating monoclonal anti-CD20 antibodies upon administration in a biological sample by using anti-idiotypic antibodies which target the anti-CD20 antibodies.

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In yet another embodiment, the human monoclonal antibodies are administered by maintenance therapy, such as, *e.g.*, once a week for a period of 6 months or more.

In still another embodiment, the human monoclonal antibodies according to the invention may be administered by a regimen including one infusion of a human monoclonal antibody against CD20 followed by an infusion of a human monoclonal antibody against CD20 conjugated to a radioisotope. The regimen may be repeated, *e.g.*, 7 to 9 days later.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in US 5,399,163; US 5,383,851; US 5,312,335; US 5,064,413; US 4,941,880; US 4,790,824; or US 4,596,556. Examples of well-known implants and modules useful in the present invention include: US 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; US 4,486,194, which discloses a therapeutic device for administering medicants through the skin; US 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; US 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; US 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and US 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the human monoclonal antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, US 4,522,811; US 5,374,548; and US 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*see, e.g.*, V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, US 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L.

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Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the desired area, *e.g.*, the site of inflammation or infection, or the site of a tumor. The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

In a further embodiment, human monoclonal antibodies of the invention can be formulated to prevent or reduce their transport across the placenta. This can be done by methods known in the art, *e.g.*, by PEGylation of the antibodies or by use of F(ab)2' fragments. Further references can be made to "Cunningham-Rundles C, Zhuo Z, Griffith B, Keenan J. (1992) Biological activities of polyethylene-glycol immunoglobulin conjugates. Resistance to enzymatic degradation. *J Immunol Methods.* 152:177-190; and to "Landor M. (1995) Maternal-fetal transfer of immunoglobulins, *Ann Allergy Asthma Immunol* 74:279-283. This is particularly relevant when the antibodies are used for treating or preventing recurrent spontaneous abortion.

A "therapeutically effective dosage" for tumor therapy can be measured by objective tumor responses which can either be complete or partial. A complete response (CR) is defined as no clinical, radiological or other evidence of disease. A partial response (PR) results from a reduction in aggregate tumor size of greater than 50%. Median time to progression is a measure that characterizes the durability of the objective tumor response.

A "therapeutically effective dosage" for tumor therapy can also be measured by its ability to stabilize the progression of disease. The ability of a compound to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit cell growth or apoptosis by *in vitro* assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

A "therapeutically effective dosage" for rheumatoid arthritis preferably will result in an ACR20 Preliminary Definition of Improvement in the patients, more preferred in an ACR50 Preliminary Definition of Improvement and even more preferred in an ARC70 Preliminary Definition of Improvement.

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ACR20 Preliminary Definition of Improvement is defined as: ≥20% improvement in: Tender Joint Count (TCJ) and Swollen Joint Count (SWJ) and ≥ 20% improvement in 3 of following 5 assessments: Patient Pain Assessment (VAS), Patient Global assessment (VAS), Physician Global Assessment (VAS), Patent Self-Assessed Disability (HAQ), Acute Phase Reactant (CRP or ESR).

ACR50 and ACR70 are defined in the same way with ≥50% and ≥70% improvements, respectively. For further details see Felson *et al.* in American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis; Arthritis Rheumatism (1995) 38: 727-735.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

VI. Uses and Methods of the Invention

The human antibodies (including immunoconjugates, bispecifics/multispecifics, compositions and other derivatives described herein) of the present invention have numerous *in vitro* and *in vivo* diagnostic and therapeutic utilities involving the diagnosis and treatment of disorders involving cells expressing CD20. For example, the antibodies can be administered to cells in culture, *e.g.*, *in vitro* or *ex vivo*, or to human subjects, *e.g.*, *in vivo*, to treat, prevent and to diagnose a variety of disorders. As used herein, the term "subject" is intended to include human and non-human animals which respond to the human antibodies against CD20. Preferred subjects include human patients having disorders that can be corrected or ameliorated by inhibiting or controlling B cells (normal or malignant).

For example, in one embodiment, human antibodies of the present invention can be used to treat a subject with a tumorigenic disorder, *e.g.*, a disorder characterized by the presence of tumor cells expressing CD20 including, for example, B cell lymphoma, *e.g.*, NHL. Examples of tumorigenic diseases which can be treated

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and/or prevented include B cell lymphoma, *e.g.*, NHL, including precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia(CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), including low-grade, intermediate-grade and high-grade FL, cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL).

Further examples of B cell non-Hodgkin's lymphomas are lymphomatoid granulomatosis, primary effusion lymphoma, intravascular large B cell lymphoma, mediastinal large B cell lymphoma, heavy chain diseases (including γ, μ, and α disease), lymphomas induced by therapy with immunosuppressive agents, such as cyclosporine-induced lymphoma, and methotrexate-induced lymphoma.

In a further embodiment, the human antibodies of the present invention can be used to treat Hodgkin's lymphoma.

Examples of immune disorders in which CD20 expressing B cells are involved which can be treated and/or prevented include autoimmune disorders, such as psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases. Further examples are severe acute respiratory distress syndrome and choreoretinitis. Furthermore, other diseases and disorders include those caused by or mediated by infection of B-cells with virus, such as Epstein-Barr virus (EBV).

Further examples of inflammatory, immune and/or autoimmune disorders in which autoantibodies and/or excessive B lymphocyte activity are prominent and which can be treated and/or prevented, include the following:

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vasculitides and other vessel disorders, such as microscopic polyangiitis, Churg-Strauss syndrome, and other ANCA-associated vasculitides, polyarteritis nodosa, essential cryoglobulinaemic vasculitis, cutaneous leukocytoclastic angiitis, Kawasaki disease, Takayasu arteritis, giant cell arthritis, Henoch-Schönlein purpura, primary or isolated cerebral angiitis, erythema nodosum, thrombangiitis obliterans, thrombotic thrombocytopenic purpura (including hemolytic uremic syndrome), and secondary vasculitides, including cutaneous leukocytoclastic vasculitis (*e.g.*, secondary to hepatitis B, hepatitis C, Waldenström's macroglobulinemia, B-cell neoplasias, rheumatoid arthritis, Sjögren's syndrome, or systemic lupus erythematosus); further examples are erythema nodosum, allergic vasculitis, panniculitis, Weber-Christian disease, purpura hyperglobulinaemica, and Buerger's disease;

skin disorders, such as contact dermatitis, linear IgA dermatosis, vitiligo, pyoderma gangrenosum, epidermolysis bullosa acquisita, pemphigus vulgaris (including cicatricial pemphigoid and bullous pemphigoid), alopecia areata (including alopecia universalis and alopecia totalis), dermatitis herpetiformis, erythema multiforme, and chronic autoimmune urticaria (including angioneurotic edema and urticarial vasculitis);

immune-mediated cytopenias, such as autoimmune neutropenia, and pure red cell aplasia;

connective tissue disorders, such as CNS lupus, discoid lupus erythematosus, CREST syndrome, mixed connective tissue disease, polymyositis/dermatomyositis, inclusion body myositis, secondary amyloidosis, cryoglobulinemia type I and type II, fibromyalgia, phospholipid antibody syndrome, secondary hemophilia, relapsing polychondritis, sarcoidosis, stiff man syndrome, and rheumatic fever; a further example is eosinophil fasciitis;

arthritides, such as ankylosing spondylitis, juvenile chronic arthritis, adult Still's disease, and SAPHO syndrome; further examples are sacroileitis, reactive arthritis, Still's disease, and gout;

hematologic disorders, such as aplastic anemia, primary hemolytic anemia (including cold agglutinin syndrome), hemolytic anemia secondary to CLL or systemic lupus erythematosus; POEMS syndrome, pernicious anemia, and Waldemström's purpura hyperglobulinaemica; further examples are agranulocytosis, autoimmune neutropenia, Franklin's disease, Seligmann's disease, μ-chain disease, paraneoplastic syndrome secondary to thymoma and lymphomas, and factor VIII inhibitor formation;

endocrinopathies, such as polyendocrinopathy, and Addison's disease; further examples are autoimmune hypoglycemia, autoimmune hypothyroidism, autoimmune insulin syndrome, de Quervain's thyroiditis, and insulin receptor antibody-mediated insulin resistance;

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hepato-gastrointestinal disorders, such as celiac disease, Whipple's disease, primary biliary cirrhosis, chronic active hepatitis, and primary sclerosing cholangiitis; a further example is autoimmune gastritis;

nephropathies, such as rapid progressive glomerulonephritis, post-streptococcal nephritis, Goodpasture's syndrome, membranous glomerulonephritis, and cryoglobulinemic nephritis; a further example is minimal change disease;

neurological disorders, such as autoimmune neuropathies, mononeuritis multiplex, Lambert-Eaton's myasthenic syndrome, Sydenham's chorea, tabes dorsalis, and Guillain-Barré's syndrome; further examples are myelopathy/tropical spastic paraparesis, myasthenia gravis, acute inflammatory demyelinating polyneuropathy, and chronic inflammatory demyelinating polyneuropathy;

cardiac and pulmonary disorders, such as fibrosing alveolitis, bronchiolitis obliterans, allergic aspergillosis, cystic fibrosis, Löffler's syndrome, myocarditis, and pericarditis; further examples are hypersensitivity pneumonitis, and paraneoplastic syndrome secondary to lung cancer;

allergic disorders, such as bronchial asthma and hyper-IgE syndrome; a further example is amaurosis fugax;

ophthalmologic disorders, such as idiopathic chorioretinitis;

infectious diseases, such as parvovirus B infection (including hands-and-socks syndrome); and

gynecological-obstretical disorders, such as recurrent abortion, recurrent fetal loss, and intrauterine growth retardation; a further example is paraneoplastic syndrome secondary to gynaecological neoplasms;

male reproductive disorders, such as paraneoplastic syndrome secondary to testicular neoplasms; and

transplantation-derived disorders, such as allograft and xenograft rejection, and graft-versus-host disease.

In one embodiment, the disease is an inflammatory, immune and/or autoimmune disorder selected from ulcerative colitis, Crohn's disease, juvenile onset diabetes, multiple sclerosis, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia (including autoimmune hemolytic anemia), myasthenia gravis, systemic sclerosis, and pemphigus vulgaris.

In another embodiment, human antibodies of the invention can be used to detect levels of CD20, or levels of cells which contain CD20 on their membrane surface, which levels can then be linked to certain disease symptoms. Alternatively, the antibodies can be used to deplete or interact with the function of CD20 expressing cells, thereby implicating these cells as important mediators of the disease. This can be

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achieved by contacting a sample and a control sample with the anti-CD20 antibody under conditions that allow for the formation of a complex between the antibody and CD20. Any complexes formed between the antibody and CD20 are detected and compared in the sample and the control.

Human antibodies of the invention can be initially tested for binding activity associated with therapeutic or diagnostic use *in vitro*. For example, the antibodies can be tested using flow cytometric assays described in the Examples below. Moreover, activity of the antibodies in triggering at least one effector-mediated effector cell activity, including inhibiting the growth of and/or killing of cells expressing CD20, can be assayed. For example, the ability of the antibodies to trigger CDC and/or apoptosis can be assayed. Protocols for assaying for CDC, homotypic adhesion, molecular clustering or apoptosis are described in the Examples below.

Human antibodies of the invention also have additional utility in therapy and diagnosis of a variety of CD20-related diseases. For example, the human antibodies can be used to elicit *in vivo* or *in vitro* one or more of the following biological activities: to inhibit the growth of and/or differentiation of a cell expressing CD20; to kill a cell expressing CD20; to mediate phagocytosis or ADCC of a cell expressing CD20 in the presence of human effector cells; to mediate CDC of a cell expressing CD20 in the presence of complement; to mediate apoptosis of a cell expressing CD20; to induce homotypic adhesion; and/or to induce translocation into lipid rafts upon binding CD20.

In a particular embodiment, the human antibodies are used *in vivo* to treat, prevent or diagnose a variety of CD20-related diseases. Examples of CD20-related diseases include, among others, B cell lymphoma, *e.g.*, NHL, and immune diseases, *e.g.*, autoimmune diseases, such as those listed above.

In a particular embodiment, the antibodies of the invention are used to treat or to prevent NHL, as the antibodies deplete the CD20 bearing tumor cells).

Non-Hodgkin's lymphoma is a type of B cell lymphoma. Lymphomas, *e.g.*, B cell lymphomas, are a group of related cancers that arise when a lymphocyte (a blood cell) becomes malignant. The normal function of lymphocytes is to defend the body against invaders: germs, viruses, fungi, even cancer. There are many subtypes and maturation stages of lymphocytes and, therefore, there are many kinds of lymphomas. Like normal cells, malignant lymphocytes can move to many parts of the body. Typically, lymphoma cells form tumors in the lymphatic system: bone marrow, lymph nodes, spleen, and blood. However, these cells can migrate to other organs. Certain types of lymphoma will tend to grow in locations in which the normal version of the cell resides. For example, it's common for follicular NHL tumors to develop in the lymph nodes.

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CD20 is usually expressed at elevated levels on neoplastic (*i.e.*, tumorigenic) B cells associated with NHL. Accordingly, CD20 binding antibodies of the invention can be used to deplete CD20 bearing tumor cells which lead to NHL and, thus, can be used to prevent or treat this disease.

Human antibodies (*e.g.*, human monoclonal antibodies, multispecific and bispecific molecules) of the present invention also can be used to block or inhibit other effects of CD20. For example, it is known that CD20 is expressed on B lymphocytes and is involved in the proliferation and/or differentiation of these cells. Since B lymphocytes function as immunomodulators, CD20 is an important target for antibody mediated therapy to target B lymphocytes, *e.g.*, to inactivate or kill B lymphocytes, involved in autoimmune disorders. Such autoimmune disorders include, for example, the above listed diseases

Suitable routes of administering the antibody compositions (*e.g.*, human monoclonal antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition. Furthermore, tumor load can be determined and used to calculate suitable dosages.

As previously described, human anti-CD20 antibodies of the invention can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The antibody can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, an anti-cancer therapy, *e.g.*, radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide. Co-administration of the human anti-CD20 antibodies of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the antibody.

Target-specific effector cells, *e.g.*, effector cells linked to compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be used as therapeutic agents. Effector cells for targeting can be human leukocytes such as macrophages, neutrophils or monocytes. Other cells include eosinophils, natural

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killer cells and other IgG- or IgA-receptor bearing cells. If desired, effector cells can be obtained from the subject to be treated. The target-specific effector cells, can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells administered can be in the order of 10^8 to 10^9 but will vary depending on the therapeutic purpose. In general, the amount will be sufficient to obtain localization at the target cell, *e.g.*, a tumor cell expressing CD20, and to effect cell killing by, *e.g.*, phagocytosis. Routes of administration can also vary.

Therapy with target-specific effector cells can be performed in conjunction with other techniques for removal of targeted cells. For example, anti-tumor therapy using the compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention and/or effector cells armed with these compositions can be used in conjunction with chemotherapy. Additionally, combination immunotherapy may be used to direct two distinct cytotoxic effector populations toward tumor cell rejection. For example, anti-CD20 antibodies linked to anti-Fc-γRI or anti-CD3 may be used in conjunction with IgG- or IgA-receptor specific binding agents.

Bispecific and multispecific molecules of the invention can also be used to modulate FcγR or FcαR levels on effector cells, such as by capping and elimination of receptors on the cell surface. Mixtures of anti-Fc receptors can also be used for this purpose.

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention which have complement binding sites, such as portions from IgG1, -2, or -3 or IgM which bind complement, can also be used in the presence of complement. In one embodiment, *ex vivo* treatment of a population of cells comprising target cells with a binding agent of the invention and appropriate effector cells can be supplemented by the addition of complement or serum containing complement. Phagocytosis of target cells coated with a binding agent of the invention can be improved by binding of complement proteins. In another embodiment target cells coated with the compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be lysed by complement. In yet another embodiment, the compositions of the invention do not activate complement.

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention can also be administered together with complement. Accordingly, within the scope of the invention are compositions comprising human antibodies, multispecific or bispecific molecules and serum or complement. These compositions are advantageous in that the complement is located in close proximity to the human antibodies, multispecific or bispecific molecules. Alternatively, the human antibodies, multispecific or bispecific molecules of the invention and the complement or serum can be administered separately. Binding of the

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compositions of the present invention to target cells causes translocation of the CD20 antigen-antibody complex into lipid rafts of the cell membrane. Such translocation creates a high density of antigen-antibody complexes which may efficiently activate and/or enhance CDC.

Also within the scope of the present invention are kits comprising the antibody compositions of the invention (*e.g.*, human antibodies and immunoconjugates) and instructions for use. The kit can further contain one or more additional reagents, such as an immunosuppressive reagent, a cytotoxic agent or a radiotoxic agent, or one or more additional human antibodies of the invention (*e.g.*, a human antibody having a complementary activity).

Accordingly, patients treated with antibody compositions of the invention can be additionally administered (prior to, simultaneously with, or following administration of a human antibody of the invention) with another therapeutic agent, such as a cytotoxic or radiotoxic agent, which enhances or augments the therapeutic effect of the human antibodies.

In other embodiments, the subject can be additionally treated with an agent that modulates, *e.g.*, enhances or inhibits, the expression or activity of Fcγ or Fcα receptors by, for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the multispecific molecule include of granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF).

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be used to target cells expressing FcγR or CD20, for example for labeling such cells. For such use, the binding agent can be linked to a molecule that can be detected. Thus, the invention provides methods for localizing *ex vivo* or *in vitro* cells expressing Fc receptors, such as FcγR, or CD20. The detectable label can be, *e.g.*, a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor.

In a particular embodiment, the invention provides methods for detecting the presence of CD20 antigen in a sample, or measuring the amount of CD20 antigen, comprising contacting the sample, and a control sample, with a human monoclonal antibody which specifically binds to CD20, under conditions that allow for formation of a complex between the antibody or portion thereof and CD20. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of CD20 antigen in the sample.

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In other embodiments, the invention provides methods for treating a disorder involving cells expressing CD20 in a subject, *e.g.*, non-Hodgkin's lymphoma or rheumatoid arthritis, by administering to the subject the human antibodies described above. Such antibodies and derivatives thereof are used to inhibit CD20 induced activities associated with certain disorders, *e.g.*, proliferation and/or differentiation. By contacting the antibody with CD20 (*e.g.*, by administering the antibody to a subject), the ability of CD20 to induce such activities is inhibited and, thus, the associated disorder is treated.

Accordingly, in another embodiment, the present invention provides a method for treating or preventing a tumorigenic disorder involving CD20 expressing cells, *e.g.*, NHL. The method involves administering to a subject an antibody composition of the present invention in an amount effective to treat or prevent the disorder. The antibody composition can be administered alone or along with another therapeutic agent, such as a cytotoxic or a radiotoxic agent which acts in conjunction with or synergistically with the antibody composition to treat or prevent the diseases involving CD20 expressing cells. In a particularly preferred embodiment, the present invention provides a method for treating non-Hodgkin's lymphoma.

In another embodiment, the present invention provides a method for treating or preventing an autoimmune disorder involving human CD20 expressing cells, *e.g.*, those diseases as listed above. The method involves administering to a subject an antibody composition of the present invention in an amount effective to treat or prevent the disorder. The antibody composition can be administered alone or along with another therapeutic agent, such as an immunosuppressant which acts in conjunction with or synergistically with the antibody composition to treat or prevent the disease involving cells expressing CD20.

In still another embodiment, the invention provides a method for detecting the presence or quantifying the amount of CD20-expressing cells *in vivo* or *in vitro*. The method comprises (i) administering to a subject a composition (*e.g.*, a multi- or bispecific molecule) of the invention conjugated to a detectable marker; (ii) exposing the subject to a means for detecting said detectable marker to identify areas containing CD20-expressing cells.

In yet another embodiment; immunoconjugates of the invention can be used to target compounds (*e.g.*, therapeutic agents, labels, cytotoxins, radiotoxins immunosuppressants, *etc.*) to cells which have CD20 expressed on their surface by linking such compounds to the antibody. Thus, the invention also provides methods for localizing *ex vivo* or *in vitro* cells expressing CD20, such as Reed-Sternberg cells (*e.g.*, with a detectable label, such as a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor). Alternatively, the immunoconjugates can be used to kill cells

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which have CD20 expressed on their surface by targeting cytotoxins or radiotoxins to CD20.

The present invention is further illustrated by the following examples which should not be construed as further limiting.

EXAMPLES

B-cell lines used in the examples

Cell line	Origin	Obtained from
Daudi	Negroid Burkitt's Lymphoma	ECACC (85011437)
ARH-77	IgG plasma cell leukemia	DSMZ (ACC 512)
DOHH	Refractory immunoblastic B cell lymphoma	DSMZ (ACC 47)
Raji	Negroid Burkitt's Lymphoma	ECACC (85011429)
SU-DHL-4	B-NHL, diffuse histiocytic lymphoma	DSMZ (ACC 495)
Ramos-EHRB	Burkitt's Lymphoma	ECACC (85030804)
Tanoue	Human B-cell leukemia	DSMZ (ACC 399)

Daudi, ARH-77, DOHH, Raji, Ramos-EHRB, and Tanoue B-cell lines were cultured in RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS) (Optimum C241, Wisent Inc., st. Bruno, Canada), 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate (all Gibco BRL, Life Technologies, Paisley, Scotland).

SU-DHL-4 B-cell line was cultured in the same medium but without sodium pyruvate.

Cultures were maintained at 37 °C in a humidified 5% CO_2 incubator, split and harvested at 80-90% confluence. Medium was refreshed twice a week. At this time cells were split and seeded out to 1-1.5 x 10^6 cells/ml to ensure viability and optimal growth.

Example 1 Production of Human Antibodies Against CD20

HCo7 and KM Mice: Fully human monoclonal antibodies to CD20 were prepared using HCo7 and KM mice which express human antibody genes. In the KM mouse strain, the endogenous mouse kappa light chain gene has been homozygously disrupted as described in Chen *et al.* (1993) *EMBO J.* 12:811-820 and the endogenous mouse heavy chain gene has been homozygously disrupted as described in Example 1 of

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PCT Publication WO 01/09187. This mouse strain carries a human kappa light chain transgene, KCo5, as described in Fishwild *et al.* (1996) *Nature Biotechnology* 14:845-851. This mouse strain also carries a human heavy chain transchromosome composed of chromosome 14 fragment hCF (SC20) as described in WO 02/43478.

The HCo7 mice have a JKD disruption in their endogenous light chain (kappa) genes (as described in Chen *et al.* (1993) *EMBO J.* 12: 821-830), a CMD disruption in their endogenous heavy chain genes (as described in Example 1 of WO 01/14424), a KCo5 human kappa light chain transgene (as described in Fishwild *et al.* (1996) *Nature Biotechnology* 14:845-851), and a HCo7 human heavy chain transgene (as described in US 5,770,429).

HCo7 and KM Mice Immunizations: HCo7 and KM mice were immunized with human CD20 transfected NS/0 cells. For the first immunization, per mouse, $1x10^7$ cells in 150 µl PBS were mixed 1:1 with Complete Freunds Adjuvant and injected intraperitoneally (i.p.). Subsequent i.p. immunizations were done using a similar amount of cells without adjuvant. Three and two days prior to fusion the mice were intravenously boosted with 0.5×10^7 cells suspended in PBS.

The presence of antibodies directed against human CD20 in the serum of the mice was monitored by flow cytometry using FACS analysis, using human CD20 transfected NS/0 cells as well as CD20 negative parental NS/0 cells.

Generation of Hybridomas Producing Human Monoclonal Antibodies to CD20: The mouse splenocytes were isolated from the HCo7 and KM mice and fused with PEG to a mouse myeloma cell line based upon standard protocols. The resulting hybridomas were then screened for human IgG,κ production by ELISA and for CD20 specificity using human CD20 transfected NS/0 and SKBR3 cells by FACS analysis. Single cell suspensions of splenic lymphocytes from immunized mice were fused to one-fourth the number of SP2/0 nonsecreting mouse myeloma cells (ATCC, CRL 1581) with 50% PEG (Sigma). Cells were plated at approximately 1×10^5/well in flat bottom microtiter plate, followed by about two week incubation in selective medium containing 10% fetal bovine serum, 10% P388D1 (ATCC, CRL TIB-63) conditioned medium, 3-5% origen (IGEN) in DMEM (Mediatech, CRL 10013, with high glucose, L-glutamine and sodium pyruvate) plus 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 mg/ml gentamycin and 1x HAT (Sigma, CRL P-7185). After 1-2 weeks, cells were cultured in medium in which the HAT was replaced with HT. Individual wells were then screened by flow cytometry for human anti-CD20 monoclonal IgG antibodies. Once extensive hybridoma growth occurred, medium was monitored usually after 10-14 days. The antibody secreting hybridomas were replated, screened again and, if still positive for

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human IgG, anti-CD20 monoclonal antibodies were subcloned by limiting dilution. The stable subclones were then cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization. One clone from each hybridoma, which retained the reactivity of parent cells (by FACS), was chosen. 5-10 vial cell banks were generated for each clone and stored in liquid nitrogen.

Selection of Human Monoclonal Antibodies Binding to CD20/Primary Screens: To determine the isotype of antibodies, an isotype ELISA was performed. Wells of microtiter plates were coated with 1 μg/ml of mouse anti-human kappa light chain, 50 μl/well in PBS incubated 4 °C overnight. After blocking with 5% chicken serum, the plates were reacted with supernatant and purified isotype control. Plates were then incubated at ambient temperature for 1-2 hours. The wells were then reacted with either human IgG1, IgG2, IgG3 or IgG4-specific Horseradish peroxidase – conjugated probes. Plates were developed and analyzed as described above.

Four hybridoma cell lines were generated, three from fusion of KM mouse and one from fusion of HCo7 mouse, expressing the following antibodies:

2F2: a human monoclonal IgG1,κ antibody with the nucleotide sequences: SEQ ID NOs: 1 and 3 and the amino acid sequences: SEQ ID NOs: 2 and 4.

4C9: a human monoclonal IgG1,κ antibody with exactly the same amino acid sequences as 2F2: SEQ ID NOs: 2 and 4.

7D8: a human monoclonal IgG1,κ antibody with the nucleotide sequences: SEQ ID NOs: 5 and 7 and the amino acid sequences: SEQ ID NOs: 6 and 8.

11B8: a human monoclonal IgG3, κ antibody with the nucleotide sequences: SEQ ID NOs: 9 and 11 and the amino acid sequences: SEQ ID NOs: 10 and 12.

The term "2F2" is used herein to designate both the antibody derived from hybridoma clone 2F2 and the identical antibody derived from hybridoma clone 4C9.

The antibodies of the invention can be switched to other isotypes as determined by the transgenic or transchromosomal non-human animal from which they are derived. In one embodiment of the invention, the 11B8 human monoclonal IgG3,κ antibody can be switched to a human monoclonal IgG1,κ isotype having exactly the same V_H and V_L sequences. In another embodiment, the 2F2 IgG1,κ antibody or 7D8 IgG1,κ antibody can be switched to a human monoclonal IgG2, IgG4, IgA1, IgA2 or IgE isotype having exactly the same V_H and V_L sequences.

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Example 2 Antibody Sequencing of Human Antibodies Against CD20

Sequencing of the V_L and V_H Regions

RNA preparation: Total RNA was prepared from $5x10^6$ cells of all HuMAb CD20 hybridoma cell lines (2F2, 7D8 and 11B8) with RNeasy kit (Qiagen, Westburg, Leusden, Netherlands) according to the manufacturer's protocol.

cDNA preparation of 2F2 and 7D8: 5'-RACE-Ready Complementary DNA (cDNA) of RNA was prepared from 1 µg total RNA, using the SMART RACE cDNA Amplification kit (Clonetech), following the manufacturer's protocol.

V_H and V_L regions were amplified using an advantage HF 2 PCR Kit (Clonetech, BD) and using the following primers:

V_K RACE2	5' GCA GGC ACA CAA CAG AGG CAG TTC CAG ATT TC	anneals in C-kappa
V_H RACE2	5' GCT GTG CCC CCA GAG GTG CTC TTG GAG G	anneals in C_{H1}

cDNA preparation of 11B8: Complementary DNA (cDNA) of RNA from 11B8 cells was prepared from 3 µg total RNA with AMV Reverse Transcriptase with buffer (Roche Diagnostics GmbH, Mannheim, Germany), oligo $d(T)_{15}$ (Promega, Madison, WI, USA), dNTP (Roche Diagnostics GmbH, Mannheim, Germany) and RNAsin (Promega) according to the manufacturer's protocol (2000, version 3).

PCR primers used to amplify V_H and V_L regions for cloning:

Primer pairs used:

V_H: FR1 5' primers

AB62	CAg gTK CAg CTg gTg CAg TC
AB63	SAg gTg CAg CTg KTg gAg TC
AB65	gAg gTg CAg CTg gTg CAg TC

V_H leader 5' primers

AB85	ATg gAC Tgg ACC Tgg AgC ATC
AB86	ATg gAA TTg ggg CTg AgC Tg
AB87	ATg gAg TTT ggR CTg AgC Tg
AB88	ATg AAA CAC CTg Tgg TTC TTC
AB89	ATg ggg TCA ACC gCC ATC CT

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V_H 3' primer

AB90	TgC CAg ggg gAA gAC CgA Tgg

V_K:

FR1 5' primers

AB8	RAC ATC CAg ATg AYC CAg TC
AB9	gYC ATC YRg ATg ACC CAg TC
AB10	gAT ATT gTg ATg ACC CAg AC
AB11	gAA ATT gTg TTg ACR CAg TC
AB12	gAA ATW gTR ATg ACA CAg TC
AB13	gAT gTT gTg ATg ACA CAG TC
AB14	gAA ATT gTg CTg ACT CAg TC

V_K leader 5' primers

AB123	CCC gCT Cag CTC CTg ggg CTC CTg
AB124	CCC TgC TCA gCT CCT ggg gCT gC
AB125	CCC AgC gCA gCT TCT CTT CCT CCT gC
AB126	ATg gAA CCA Tgg AAg CCC CAg CAC AgC

V_K 3' primer

AB16	Cgg gAA gAT gAA gAC AgA Tg

wherein K = T or G, S = C or G, R = A or G, Y = C or T, and W = A or T.

PCR conditions used to amplify V_H and V_L regions for cloning 2F2 and 7D8: Polymerase chain reactions (PCR) were performed with HF polymerase mix (Clonetech.) on a T1 cycler (Biometra, Westburg).

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PCR conditions:

94 °C 30 sec 5 cycles
72 °C 1 min

94 °C 30 sec
70 °C 30 sec 5 cycles
72 °C 1 min

94 °C 30 sec
68 °C 30 sec 27-30 cycles
72 °C 1 min

PCR conditions used to amplify V_H and V_L regions for cloning 11B8: Polymerase chain reactions (PCR) were performed with AmpliTaq polymerase (Perkin Elmer) on a T1 Cycler (Biometra, Westburg, Leusden, Netherlands).

PCR cycling protocol:

94 °C 2 min
11 cycles 94 °C 30 sec
65 °C 30 sec, minus 1 °C per cycle
72 °C 30 sec
30 cycles 94 °C 30 sec
55 °C 30 sec
72 °C 30 sec
72 °C 10 min
cool down to 4 °C

Cloning of V_H and V_L in pGEMT-Vector System II (2F2, 7D8, and 11B8): After analysing the PCR products on an agarose gel, the products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Westburg, Leusden, Netherlands). Two independently amplified PCR products of each V_H and V_L region were cloned in pGEMT-Vector System II (Promega) according to manufacturer's protocol (1999, version 6).

After transformation to *E. coli* JM109, individual colonies were screened by colony PCR using T7 and SP6 primers, 30 annealing cycles at 55 °C. Plasmid DNA from colonies was purified using Qiaprep Spin miniprep kit (Qiagen). To further

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analyse the V_H and V_L regions, a *Nco*1/*Not*1 (NE Biolabs, Westburg, Leusden, Netherlands) digestion was performed and analysed on agarose gel.

Sequencing (2F2, 7D8 and 11B8): The V-regions regions were sequenced after cloning in the pGEMT-Vector System II. Sequencing was performed at Baseclear (Leiden, Netherlands). The sequences were analyzed by aligning germline V-gene sequences in Vbase (www.mrc-cpe.cam.ac.uk/imt-doc/public/intro.htm).http://www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901.

The sequences obtained are shown in Figures 53-58.

Example 3 Recombinant production of 2F2 and 11B8 in GS-NS/0 cell line

2F2T: The heavy chain and light chain variable regions of the 2F2 antibody were amplified, using PCR, from a standard cloning vector, pGem-5Zf (Promega), using primers which included an optimal Kozak sequence and suitable restriction sites to clone the fragments in the GS constant region vectors pCONγ1f and PCONκ (Lonza).

After amplification, the fragments were purified and digested with the restriction enzymes for cloning and ligated in the two vectors. The heavy chain variable fragment was digested with *Hind* III and *Bsi* WI and ligated into the pCONγ1f vector which had been digested with *Hind* III and *Bsi* WI, and dephosphorylated with alkaline phosphatase. The light chain variable fragment was digested with *Hind* III and *Apa* I and ligated into the PCONκ vector which had been digested with *Hind* III and *Apa* I, and dephosphorylated with alkaline phosphatase. The pCONγ1f/variable-heavy and PCONκ/variable-light vectors are shown in Figures 1 and 2, respectively. Transformed *E. coli* colonies were checked by colony PCR and 2 positive colonies of each the heavy chain (HC) and light chain (LC) construct were grown for plasmid isolation. Isolated plasmid of these 4 clones was sequenced to confirm the sequence. Both of the HC clones and one of the LC clones were found to have the correct sequences.

The two HC and one LC constructs were combined to give two combinations of LC-HC and transiently co-transfected in CHO-K1 cells to check the constructs for proper production of 2F2 antibody. Normal production levels were reached for all combinations in this expression experiment and 1 clone of each of the HC and LC constructs were chosen for construction of a double-gene vector.

Standard cloning procedures were used to combine the HC and LC constructs in a double-gene cloning vector, designated pCONγ1f/κ2F2, by ligating the complete expression cassette from the heavy chain vector, pCONγ1f/variable-heavy,

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into the light chain vector, pCONκ/variable-light. The pCONγ1f/κ2F2 vector is shown in Figure 3.

This construct was again functionally tested in a transient transfection in CHO-K1 cells and showed normal expression levels.

The variable regions of the pCONγ1f/κ2F2 plasmid were sequenced to reconfirm the correct sequences.

Linear plasmid was prepared for stable transfections by digesting pCONγ1f/κ2F2 with a unique restriction enzyme, *Pvu I,* cutting outside regions vital for expression. Complete linearization was confirmed by agarose gel electrophoresis and the DNA was purified and stored at -20 °C until use.

Six transfections of NS/0 host cells were performed, by electroporation with plasmid DNA, using the above linear DNA plasmid. Following transfection, the cells were distributed into 96-wells plates and incubated. Selective medium (containing 10% dialysed fetal calf serum (dFCS) and 10 μM of the GS-inhibitor L-methionine sulphoximine but lacking glutamine) was added and the plates were monitored to determine when the non-transfected cells died to leave foci of transfected cells. For further details concerning GS vector systems, see WO 87/04462. The transfected plates were incubated for approximately three weeks to allow colony formation. The resulting colonies were examined microscopically to verify that the colonies were of a suitable size for assay (covering greater than 60% of the bottom of the well), and that only one colony was present in each well. Cell supernatants from 436 transfectants were screened for assembled antibody by IgG,κ-ELISA. Using this data, 111 transfectants were selected for progression and further assessment in static culture. Cultures of the selected cell lines were expanded and adapted to low-serum containing medium (containing bovine serum albumin (BSA) and added 1% dFCS) and a further assessment of productivity in static culture was undertaken (ELISA and measurement of percentage confluence). The 65 highest ranking cell lines were selected for progression. A preliminary assessment of the productivity of the selected cell lines was made in batch shake flask suspension culture in low serum-containing medium (containing BSA and added 1% dFCS). Based upon harvest antibody concentration (by ELISA) and acceptable growth characteristics, 30 cell lines were selected for further evaluation in serum-free medium using a batch shake flask suspension culture. The 10 cell lines that produced the highest antibody concentrations were further evaluated in duplicate fed-batch shake flask suspension cultures in serum-free medium. Product concentrations at harvest were determined by protein A high performance liquid chromatography (HPLC), according to well-known standard methods. All cell lines produced 2F2 antibody (denoted 2F2T) in good yields in the range of from 671-1333 mg/L as determined by protein A HPLC.

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11B8T: In a similar way a GS-NS/0 cell line was established for recombinant production of 11B8 (denoted 11B8T) modifying the transfection procedure slightly as follows.

Four transfections of NS/0 host cells were performed, by electroporation with plasmid DNA, using the above linear DNA plasmid. After examining the resulting colonies microscopically to verify that the colonies were of a suitable size for assay (covering greater than 60% of the bottom of the well) and that only one colony was present in each well, cell supernatants from 596 transfectants were screened for assembled antibody by IgG,κ-ELISA. Using this data, 100 transfectants were selected for progression and further assessment in static culture. Cultures of the selected cell lines were expanded and adapted to low-serum containing medium (containing bovine serum albumin (BSA) and added 1% dFCS) and a further assessment of productivity in static culture was undertaken (ELISA and measurement of percentage confluence). The 60 highest ranking cell lines were selected for progression, and an additional 13 cell lines for which productivity data was unavailable were also progressed. A preliminary assessment of the productivity of the selected cell lines was made in batch shake flask suspension culture in low serum-containing medium (containing BSA and added 1% dFCS). Based upon harvest antibody concentration (by ELISA) and acceptable growth characteristics, 10 cell lines were selected for further evaluation in duplicate fed-batch shake flask suspension cultures in low serum-containing medium (containing BSA and added 1% dFCS). Product concentrations at harvest were determined by protein A high performance liquid chromatography (HPLC), according to well-known standard methods. Based on this one of the cell lines was discarded. The resulting 9 cell lines all produced 11B8 antibody (denoted "11B8T") in good yields in the range of from 354-771 mg/L as determined by protein A HPLC.

Example 4 Comparison of hybridoma-derived 2F2 and transfectoma-derived recombinant 2F2T

By use of gel electrophoresis (SDS-PAGE and native agarose gel electrophoresis) it was shown that 2F2 and 2F2T are of the same size, and only slightly differ in electric charge.

Furthermore, 2F2 and 2F2T bind to CD20-transfected NS/0 cells and Raji cells with similar affinity as measured by flow cytometry using FACScalibur™ (Becton Dickinson, San Diego, CA, USA). No binding to non-transfected NS/0 cells was observed demonstrating the specificity of 2F2 and 2F2T. 2F2 and 2F2T also induce CDC in a concentration-dependent manner to the same extent in ARH-77 cells (IgG plasma cell leukemia), Daudi cells, DOHH cells (refractory immunoblastic B cell

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lymphoma progressed from follicular centroblastic/centrolytic lymphoma, DSMZ, Braunschweig, Germany) and Raji cells, measuring cell lysis (number of PI-positive cells) by flow cytometry (FACScalibur). In a second experiment, the concentrations of 2F2 and 2F2T were kept constant while serum was added in different concentrations. No significant differences between 2F2 and 2F2T were observed.

Finally, 2F2 and 2F2T bound to cell-associated CD20 binds complement factor C1q strongly and to the same extent. The experiment was performed in Daudi cells, DOHH cells, and Raji cells using fluorescein-conjugated anti-C1q polyclonal antibodies for detecting binding of C1q.

Example 5 Binding Characteristics of Human Antibodies Against CD20

Binding to different cell lines: NS/0, NS/0 transfected with human CD20, Daudi and Raji cells were incubated for 30 min at 4 °C with culture supernatant containing human antibodies 2F2, 7D8, and 11B8 followed by incubation with FITC-conjugated anti-human IgG Ab. Binding was assessed by flow cytometry using a FACScalibur flow cytometer. Fluorescence intensities were compared with negative control isotype matched samples. As shown in Figure 4, all three antibodies bound to NS/0 cells transfected with human CD20, whereas no binding was observed to parental, non-transfected NS/0 cells. All three antibodies also bound to the two different Burkitt lymphoma B cell lines (Raji and Daudi) indicating that 2F2, 7D8, and 11B8 are CD20 specific. Supernatant containing 7D8 or 11B8 were tested under non-saturating conditions, therefore, lower mean fluorescence intensities compared to 2F2 are observed.

EC_{50} value of 2F2 as determined by flow cytometry: In order to determine the apparent affinity of 2F2 for CD20 expressed on human B cells, a binding curve was made of 2F2 using isolated PBMCs from three human donors and gating of CD3-negative cells. The isolated PBMCs were incubated for 1 hour with a concentration range of FITC labeled 2F2 and analysed on FACS, and the mean fluorescence intensity (MFI) determined. The MFI values are shown in Figures 5A and 5B as a function of the antibody concentration. The EC_{50} values were calculated by use of Graph Pad Prism 3.02 by non-linear regression. The EC_{50} value of 2F2 in humans was similar for all three donors with a mean (± s.e.m.) of 287 ± 12.7 ng/ml (1.9 ± 0.1 nM).

Binding of ^{125}I-labeled mAbs to CD20 expressing cells: mAbs were iodinated using Iodobeads (Pierce Chemical Co., Rockford, IL). ^{125}I-labelled mAbs were serially diluted and incubated with Ramos-EHRB (cells for 2 hours at 37 °C in the

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presence of sodium azide and 2-deoxyglucose to prevent endocytosis. The cell bound and free ^{125}I labeled mAbs were then separated by centrifugation at 14,000 x g for 2 min through a mixture of phthalate oils, allowing rapid separation without disturbing the binding equilibrium. The pelleted cells together with bound antibody were then counted using a gamma counter (Wallac UK Ltd, Milton Keynes, UK).

As shown in Figure 6, 2F2 and 11B8 exhibit similar K_D (or similar saturation points) indicating that both antibodies bind with similar affinity. However, 11B8 saturates at a lower level than 2F2 indicating that it recognized a different form of CD20. This is also in agreement with a further experiment showing that a similar number of 2F2 and rituximab antibody molecules binds to CD20 on Ramos-EHRB cells and Daudi cells, as shown by the similar levels of binding saturation (approximately 2-3 x 10^5 antibody molecules per cell). 11B8 and B1, in contrast, saturate at half this level and only about 1-2 x 10^5 antibody molecules bind to the Ramos-EHRB cells (Figure 7A) and Daudi cells (Figure 7B).

To exclude the possibility that the iodinated antibodies bind via Fc-receptors, binding curves were confirmed by use of anti-CD20 $F(ab')_2$ fragments. Again, similar numbers of 2F2 and rituximab-$F(ab')_2$ fragments bound to both Ramos-EHRB and Daudi cells. Also in these experiments, the number of 2F2 or rituximab antibody molecules bound to Ramos-EHRB cells and Daudi cells saturates at approximately twice the number of 11B8 and B1 molecules bound to the cells.

Dissociation rate: To determine the dissociation rate of the mAbs, Ramos-EHRB cells (final volume of 1 ml in the presence of azide/2DOG) were incubated for 2 hours at 37 °C with 2 μg/ml ^{125}I mAbs to achieve maximum binding. Following centrifugation in a microfuge (2000 rpm for 2 min), the supernatant was removed, the pellet quickly resuspended in 1 ml medium, and immediately transferred to 9 ml medium at 37 °C in a 15 ml conical tube. At various times over the next 2 hours, 0.4 ml samples were removed and separated on phthalate oils to determine the level of radiolabeled mAbs remaining on the cell surface. As shown in Figure 8, both 2F2 and 11B8 dissociated significantly more slowly from CD20 than rituximab or B1.

Dissociation rates of anti-CD20 $F(ab)_2$ fragments: Ramos-EHBR cells were saturated with 2 μg/ml of ^{125}I-labeled $F(ab)_2$ fragments of 2F2, 11B8, and rituximab, respectively. The Ramos-EHBR cells were washed and incubated in the presence of a high concentration of the unlabeled antibody. The maximal (initial) binding to Ramos-EHRB cells was set at 100%. At several time points over the next 3 hours following loading, 0.4 ml samples were removed and separated on phthalate oil to determine the levels of radiolabeled mAb remaining on the cell surface. As can be seen

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from Figure 9, 2F2 and 11B8 dissociated much more slowly from the surface of CD20 than rituximab. At 90 min, approximately 50% of the $F(ab)_2$ rituximab molecules were bound to the cell, whereas half of the $F(ab)_2$ 2F2 molecules were dissociated after 3 hours. The k_d (k_{off}) values for 2F2, 11B8T, and rituximab are calculated as follows:

$F(ab)_2$ 2F2: $k_d = \ln 2/t_{½}$ (sec) = ln 2/10800 (sec) = 6.4×10^{-5} sec^{-1}

$F(ab)_2$ 11B8T: $k_d = \ln 2/t_{½}$ (sec) = ln 2/9000 (sec) = 7.7×10^{-5} sec^{-1}

$F(ab)_2$ rituximab: $k_d = \ln 2/t_{½}$ (sec) = ln 2/5400 (sec) = 1.3×10^{-4} sec^{-1}

Anti-CD20 mAb functional off rates: The impact of the slow 2F2 dissociation rate compared to rituximab was assessed in a functional CDC assay. To this end, Daudi or SU-DHL4 cells were pre-incubated with 10 μg/ml anti-CD20 mAb or an isotype control antibody, washed and incubated in medium for different time points. At these time points after start of the assay, samples were incubated with complement (normal human serum 20 vol/vol%) and then incubated for another 45 min at 37 °C. Thereafter, cell lysis was determined on FACS by using PI (propidium iodide) staining method. The % lysed cells (PI-positive cells) are shown in Figure 10A (Daudi cells) or Figure 10B (SU-DHL4 cells) as a function of incubation time. 2F2 induced high CDC in both cell lines, and still lysed up to 90% of the cells after 6 hours, indicating that the CD20 saturation of the cells remained sufficiently high to induce complement-mediated lysis of most of the cells. Rituximab, in contrast and in agreement with the above dissociation rate studies, dissociated rapidly from the cells and failed to induce specific lysis following the 6 hour incubation period. 11B8 was used as a control and did not induce CDC.

Example 6 CDC of Human Antibodies Against CD20

Serum preparation: Serum for complement lysis was prepared by drawing blood from healthy volunteers into autosep gel and clot activator vacutainer tubes (BD biosciences, Rutherford, NJ) which were held at room temperature for 30-60 min and then centrifuged at 3000 rpm for 5 min. Serum was harvested and stored at -80 °C.

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Flow cytometry: For flow cytometry a FACScalibur flow cytometer was used with CellQuest pro software (BD Biosciences, Mountain view, CA). At least 5000 events were collected for analysis with cell debris excluded by adjustment of the forward sideward scatter (FCS) threshold.

CDC kinetics: In a first set of experiments (n=3) the kinetics of CDC of five different B-cell lines, *i.e.*, Daudi, SU-DHL-4, Raji, DOHH and ARH-77, were determined by adding 10 μg/ml 2F2, rituximab and an IgG control antibody, respectively, for 10 min before human serum was added. At several time intervals (up to one hour) after induction of CDC, the cells were suspended in PI solution and cell lysis (number of PI-positive cells) was measured by flow cytometry. The results are depicted in Figures 11A (ARH-77 cells), 11 B (Daudi cells), 11C (Raji cells), 11D (DOHH) and 11 E (SU-DHL-4). As seen, addition of antibodies induced cell lysis within 5 min. Interestingly, addition of 2F2 resulted in a marked cell lysis of more than 80% in all five B-cell lines. Rituximab induced more than 80% cell lysis only in the SU-DHL-4 and Daudi cell lines, whereas the cell lysis of the DOHH cell line was ~50%, and less than 20% in the ARH-77 and Raji cell lines. No lysis was observed with the IgG control antibody (data only shown in Figure 11B).

CDC serum titration: In a separate set of experiments (n=5), NHS (normal human serum) was titrated at two different antibody concentrations of 0.5 μg/ml and 5 μg/ml. Cells were pre-incubated with 2F2 or rituximab for 10 min, before a concentration range of NHS was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 12A-D show the percentage of lysed (PI-positive) cells as a function of NHS concentration. Figure 12A shows cell lysis of Daudi cells, Figure 12B cell lysis of ARH-77 cells, Figure 12C cell lysis of DOHH cells, and Figure 12D cell lysis of Raji cells. Increased lysis of cells was observed with increased NHS concentration. Addition of 2F2 caused maximal lysis of Daudi cells at the highest NHS and antibody concentration. Rituximab induced about 50% cell lysis of Daudi cells at the highest NHS concentration.

In ARH-77 cells, only the highest concentration of NHS and 2F2 led to approximately 75% cell lysis. Lower antibody concentrations were insufficient to induce ARH-77 cell lysis. Rituximab was not able to induce cell lysis of ARH-77 cells in this experiment.

2F2 was able to induce NHS-concentration dependent cell lysis of DOHH cells at both the high and the low concentration, whereas rituximab was not able to induce lysis under these conditions.

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Finally, 2F2 induced NHS-concentration-dependent lysis of Raji cells, which was only apparent by use of 5 μg/ml mAb. No lysis was observed with rituximab.

In these experiments, no lysis was observed with the isotype control antibody (data not shown).

CDC antibody titration: To measure the ability of the anti-CD20 antibodies to induce CDC at low concentrations, an experiment was performed where the antibodies were titrated (n=6). Various cell lines were pre-incubated with a concentration range of 2F2 and rituximab, respectively, for 10 min before NHS was added. After 45 min incubation at 37 °C (when maximal lysis occurs) the cells were resuspended in PI solution and cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 13A (Daudi cells), 13B (DOHH cells), 13C (ARH-77 cells), and 13D (Raji cells) show the percentage of lysed (PI-positive) cells as a function of antibody concentration. Both 2F2 and rituximab induced a concentration-dependent increase in cell lysis. 2F2 induced more than 80% lysis of Daudi cells upon addition of 2 μg/ml, whereas with rituximab this level was not reached even after addition of 10 μg/ml. Furthermore, 2F2 induced more than 80% lysis of DOHH cells at 0.4 μg/ml, whereas minimal lysis was observed with rituximab at this concentration. The maximal lysis of DOHH cells with rituximab (~30% of total cell analyzed) was reached at 10 μg/ml. Induction of lysis of ARH-77 and Raji cells by 2F2 was lower, but still ~70% lysis was reached at an antibody concentration of 10 μg/ml. At its highest concentration, rituximab induced lysis in only ~23% of ARH-77 cells, and in only ~6% of Raji cells.

In a similar experiment, 2F2, 2F2T, 11B8T, and rituximab were investigated for their ability to induce CDC of Daudi and Raji cell lines, see Figures 14A and 14B. Also in this experiment more than 80% lysis of Daudi cells was observed with (transfectoma-derived) 2F2T at 10 μg/ml, whereas rituximab reached only to 60% lysis even at 10 μg/ml, cf. Figure 14A. Lysis of Daudi cells with 2F2T was identical to the lysis obtained with hybridoma-derived 2F2.

Lysis of Raji cells was more difficult, but again both 2F2 and 2F2T induced lysis of Raji cells to a similar extent (Figure 14B). Rituximab was not able to induce CDC of Raji cells which is in agreement with the experiment shown in Figure 13D.

As can be seen from Figures 14A and 14B neither Daudi nor Raji cells were susceptible to CDC by 11B8T. B1 induced lysis of Daudi cells, but only to a small extent, and was not able to induce lysis of Raji cells.

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CDC activity of anti-CD20 in Daudi cells: To determine the CDC activity of each antibody, elevated membrane permeability was assessed using FACS analysis of propidium iodide (PI)-stained cells. Briefly, the Daudi cells were washed and resuspended in RPMI/1% BSA at 1 x 10^6 cells/ml. Various concentrations of human monoclonal antibodies were added to the Daudi cells and allowed to bind to CD20 on the cells for 10-15 min at room temperature. Thereafter, serum as a source of complement was added to a final concentration of 20% (v/v) and the mixtures were incubated for 45 min at 37 °C. The cells were then kept at 4 °C until analysis. Each sample (150 µl) was then added to 10 µl of PI solution (10 µg/ml in PBS) in a FACS tube. The mixture was assessed immediately by flow cytometry. As shown in Figure 15A, 2F2 and 7D8 showed superior CDC activity compared to rituximab.

In a second experiment, cells were labeled with human monoclonal antibodies as above, then washed and incubated in PBS for 45 min at 37 °C prior to the addition of human serum. This ensured that only antibody bound to the cell at the time of serum addition was available to activate complement for cell lysis. As shown in Figure 15B, decreased CDC activity was found for rituximab compared to 2F2 and 7D8 indicating that the human antibodies (2F2 and 7D8) are not affected by washing the cells prior to the addition of serum.

CDC activity of anti-CD20 in Raji cells: CDC activity was assessed using Raji cells which have relatively high surface expression of CD55 and CD59 and, therefore, are more resistant to complement attack. Human antibodies were added to Raji cells and allowed to bind for 15 min. Human serum (20%) was added and the mixtures incubated for 45 min at 37 °C. As shown in Figure 16A, rituximab was ineffective in mediating CDC of Raji cells whereas significant levels of cell lysis occurred in Raji cells opsonized with 2F2 or 7D8. Accordingly, 2F2 and 7D8 have a unique capacity to lyse CD55/59 positive target cells.

In a separate experiment, Raji cells were pre-incubated with saturating concentrations of anti-CD55 mAb (final concentration of 5 µg/ml) and anti-CD59 mAb (final concentration of 5 µg/ml) to block the effects of these complement defense molecules. Human anti-CD20 antibodies were then added along with serum (20%) as above for 45 min at 37 °C. As shown in Figure 16B, the blockade of CD55 and CD59 molecules resulted in almost 100% lysis of Raji cells with human antibodies 2F2 or 7D8 whereas only a 25% increase in cell lysis was observed using rituximab.

Role of complement inhibitors I – Expression of surface molecules: Since complement inhibitors such as CD55 and CD59 appear to play an important role in susceptibility to rituximab-induced CDC, an experiment was performed to determine

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the expression of these molecules on the B-cell lines under investigation (Raji, Daudi, DOHH, ARH-77, and SU-DHL-4).

The cells were stained with FITC-conjugated anti-CD55, anti-CD59 and anti-CD20 antibodies and molecules expression was analyzed by flow cytometry. The results are shown in the below Table 1.

Table 1

Expression	CD20	CD55	CD59
ARH-77	++	++++	++
Raji	+	++	+++
DOHH	++	+++	++
SU-DHL-4	+++	+	++
Daudi	++	+	+

Role of complement inhibitors II – Blockade of CD55 and CD59: To further study the roles of CD55 and CD59 in anti-CD20-induced CDC, both complement inhibitor molecules were blocked by specific antibodies prior to induction of CDC (n=3). Raji cells were used because only partial lysis was induced by 2F2 alone. Raji cells (1 x 10^5 cells/50 µl) were pre-incubated with a concentration range of 2F2 and rituximab together with anti-CD55 (5 µg/ml) or anti-CD59 (5 µg/ml) antibodies for 10 min, before pooled NHS (20%) was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 17A-C show the percentage of lysed (PI-positive) cells as a function of antibody concentration, and show one experiment which is exemplary of three experiments. Figure 17A shows incubation of Raji cells with anti-CD55 antibody, Figure 17B incubation of Raji cells with anti-CD59 antibody, and Figure 17C incubation of Raji cells with anti-CD55 and anti-CD59 antibodies.

As can be seen in Figure 17A, addition of anti-CD55 antibody did not influence 2F2 or rituximab-induced CDC. Addition of anti-CD59 antibody increased susceptibility of the cells to both 2F2 and to rituximab with ~30% (Figure 17B). Addition of both anti-CD55 and anti-CD59 further enhanced anti-CD20-induced lysis of cells with ~30% (Figure 17C).

Role of complement factors, as determined by flow cytometry I – C1q binding: Anti-CD20 antibodies (2F2 and rituximab) and an isotype control antibody were added to various B-cell lines. After 10 min incubation, NHS (1 vol/vol%) was added. After further incubation for 10 min at 37 °C and washing of the cells, the supernatant was discarded and the cell pellet was incubated with FITC-conjugated anti-

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C1q antibody. Data show mean fluorescence intensity of cells stained with C1q and are depicted in Figures 18A (Daudi), 18B (ARH-77), 18C (DOHH), and 18D (Raji) (n=6). The results indicated antibody concentration-dependent increase in binding of C1q by 2F2, irrespective of the B-cell line investigated. Moreover, C1q binding by 2F2 was always higher than binding by rituximab, in all cell lines tested. No increase in mean fluorescence was observed with the isotype control antibody (data not shown).

Role of complement factors, as determined by flow cytometry II – Complement activation via the classical route: Fixation of C4c to antibody-coated cells is an indication of activation of complement activation via the classical route. Anti-CD20 antibodies (2F2 and rituximab) and an isotype control antibody were added to various B-cell lines. After 10 min incubation at 37 °C, NHS (1 vol/vol%) was added. After further incubation and washing of the cells, the supernatant was discarded and the cell pellet was incubated with FITC-conjugated anti-C4c antibody. Data show mean fluorescence intensity of cells stained with C4c and are depicted in Figures 19A (Daudi), 19B (ARH-77), 19C (DOHH), and 19D (Raji) (n=6). Complement factor C4c fixation to 2F2 was demonstrated in all B-cell lines tested (n=3), with a maximum reached at ~1 µg/ml of antibody. Fixation of C4c after 2F2 binding was much higher than after rituximab, irrespective of the cell line tested. No increase in mean fluorescence was observed with the isotype control antibody (data not shown).

CDC in heat-inactivated serum: Cells (Daudi cells, ARH-77 cells or Raji cells) and antibodies (rituximab, 2F2, 2F2T, 11B8, and isotype control antibody HuMab-KLH IgG1) were pre-incubated in a concentration range of anti-CD20 antibodies for 10 min, before NHS (active or heat-inactivated in a water bath at 57 °C at 30 min) was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. No lysis of the cells was observed in the presence of heat-inactivated serum, irrespective of the cell-line and CD20-antibody used, no CDC was observed in the presence of heat-inactivated serum.

Example 7 ADCC of Human Antibodies Against CD20 ADCC Assay I

Enrichment of human neutrophils: Polymorphonuclear cells (neutrophils, PMNs) were enriched from heparinized whole blood. Blood was diluted twice in RPMI 1640 and was layered on Ficoll (Lymphocyte Separation Medium 1077 g/ml, 710 g, RT, 20 min; BioWhittaker, cat. 17-829E, lot no. 0148 32) and centrifuged at 2000 rpm for 20 min. The mononuclear cell layer was removed, and erythrocytes within the pellet

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containing neutrophils were hypotonically lysed using ice-cold NH_4Cl solution (155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). The remaining neutrophils were washed twice and resuspended in RPMI 1640 supplemented with 10% FCS (RPMI-10).

Enrichment of human peripheral blood mononuclear cells: Human blood was diluted twice in RPMI 1640 and blood cells were layered on Ficoll (Lymphocyte Separation Medium 1077 g/ml, 710 g, RT, 20 min; BioWhittaker, Cambrex Bio Science Verviers, Verviers, Belgium, cat. 17-829E, lot no. 0148 32). Peripheral blood mononuclear cells (MNCs) were collected from the interphase, washed and resuspended in RPMI 1640 culture medium supplemented with 10% FCS, 2 mM L-glutamine, 5 U/ml penicillin, 50 µg/ml streptomycin (all derived from BioWhittaker) to which 25 mM HEPES (BioWhittaker) was added.

ADCC set up: Target B-cells (freshly isolated B-cells or from B-cell lines) were labeled with 20 µCi ^{51}Cr (Amersham Biosciences, Uppsala, Sweden) for 2 hours. After extensive washing in RPMI-10, the cells were adjusted to 1 x 10^5 cells/ml. Whole blood or isolated effector cells (50µl; MNCs, PMNs) or plasma (50 µl), sensitizing antibodies (50 µl), and RPMI-10 (50 µl) were added to round-bottom microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Assays were started by adding target cells (50 µl) giving a final volume of 200 µl. For isolated effector cells, an effector to target (E:T) ratio of 40:1 was used. For whole blood, an amount of 33 vol/vol% was used corresponding to an estimated effector to target ratio of 40:1. After incubation (3 hours, 37 °C), assays were stopped by centrifugation, and ^{51}Cr release from triplicates was measured in counts per minute (cpm) in a scintillation counter. Percentage of cellular cytotoxicity was calculated using the following formula:

% specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) x 100

with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release measured in the absence of sensitizing antibodies and effector cells.

Statistics: Data were analyzed by one-way ANOVA, followed by Tukey's multi comparison post-hoc test. Analysis was performed using Graph Pad Prism (version 3.02 for Windows, Graph Pad Software, San Diego, CA, USA).

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Lysis of ARH-77 cells: In a first set of experiments, ARH-77 cells were used as target cells (Figure 20). Addition of 2F2 (n=3), rituximab (n=3) or 11B8T (n=1) resulted in MNC-mediated lysis of ARH-77 cells of approximately 50%. No specific lysis was observed in the presence of neutrophils. Addition of plasma (to evaluate the role of complement) induced lysis of ARH-77 cells after incubation with 2F2, but not after incubation with rituximab ($p<0.05$, 2F2 vs. no antibody, ANOVA) or 11B8T. In the presence of whole blood, lysis of ARH-77 cells increased after incubation with 2F2 ($p<0.05$, 2F2 vs. rituximab and 2F2 vs. no antibody, ANOVA), but not with rituximab. Specific lysis induced by rituximab was in fact very low in the presence of whole blood. 11B8T induced cell lysis of approximately 25% (n=1) in the presence of whole blood. In the absence of antibody, non-specific lysis of 10-15% was observed.

Lysis of B-CLL cells: In a second set of experiments, chronic B-lymphocytic leukaemia (B-CLL) cells obtained from B-CLL patients (n=12) were subcloned for 5 rounds and then used as target cells in the experiment (Figure 21). In the absence of antibody, no specific lysis was observed, but addition of 2F2, 11B8T or rituximab (10 μg/ml) increased MNC-mediated specific lysis to 10-20% ($p<0.001$, ANOVA). Incubation of target cells with plasma and 2F2 induced specific lysis of B-CLL cells, whereas no specific lysis was observed with 11B8T or rituximab ($p<0.001$, ANOVA). Moreover, 2F2 mediated specific lysis of B-CLL cells after incubation in whole blood. No specific lysis of B-CLL cells by whole blood was observed with 11B8T ($p<0.01$, ANOVA) or rituximab ($p<0.001$, ANOVA). No specific lysis was observed in the presence of neutrophils.

Because rituximab was able to mediate effective ADCC but not CDC of the tumor cells tested, it is likely that whole blood-induced B-cell lysis by 2F2 is mediated via complement.

Lysis of hairy cell leukaemia (HCL) cells: In a third set of experiments lysis of HCL cells by 2F2, 11B8T, and rituximab by ADCC or in the presence of plasma or whole blood was determined. Data are shown in Figure 22. Whereas neutrophils could not mediate ADCC irrespective of the mAb used, 11B8T was able to induce MNC-mediated lysis of HCL cells more efficiently than 2F2 ($p<0.001$, ANOVA) or rituximab ($p<0.05$, ANOVA). 2F2 and rituximab were not able to induce MNC-mediated lysis of HCL cells. Plasma-mediated lysis of the cells was strongly enhanced with 2F2, as compared to rituximab ($p<0.05$, ANOVA), 11B8T ($p<0.01$, ANOVA) or without antibody ($p<0.001$, ANOVA). When lysis induced by anti-CD20 in the presence of whole blood was studied, 2F2 induced complete lysis of cells, and was

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superior to rituximab ($p<0.01$, ANOVA), 11B8T or no antibody added ($p<0.001$, ANOVA).

Lysis of B-ALL cells: Using cells from two patients the ability of 2F2 and rituximab to induce lysis B-ALL cells by ADCC or complement was investigated (Figure 23). As was observed in the previous experiments, 2F2 and rituximab induced MNC-mediated ADCC of B-ALL cells to a similar extent. But again 2F2 was able to induce plasma- and whole blood-mediated lysis of B-ALL cells, whereas rituximab was not.

Lysis of follicular lymphoma cells: When lysis of follicular lymphoma cells (n=2) was investigated, a different picture emerged (Figure 24). A minor PMN-mediated lysis of cells with 2F2 was observed, and both 2F2 and rituximab were not able to induce MNC-mediated ADCC. 11B8T was still able to induce MNC-mediated lysis of approximately 20%. Although a relatively high plasma-mediated lysis was induced by rituximab, complete plasma-mediated lysis was observed with 2F2. Also with whole blood, complete lysis was observed with 2F2, whereas 70% lysis with rituximab. Minimal plasma- or whole blood-mediated lysis by 11B8T was observed.

Lysis of primary mantle cell lymphoma cells: Specific lysis of mantle cell lymphoma cells was more difficult to induce (n=1, Figure 25). Minimal or no lysis by 2F2, 11B8T or rituximab was observed after addition of PMN or MNC and CD20 mAbs. However, 2F2 was still able to induce approximately 40% lysis by plasma or whole blood, whereas with rituximab only 10-20% of the cells were lysed. 11B8T was not able to induce lysis of primary mantle cell lymphoma cells.

Antibody concentration-dependent lysis of ARH-77 cells in whole blood: In a further experiment (n=4) dose-dependency regarding the induction of ADCC on ARH-77 cells in the presence of whole blood was analyzed. As can be seen in Figure 26, titration of 2F2 induced a dose-dependent increase in the percentage of specific lysis ($p<0.05$: treatment-effect, two-way ANOVA) of ARH-77 cells. No specific lysis of ARH-77 cells was observed with rituximab.

ADCC assay II

Preparation ^{51}Cr-labeled target cells: ARH-77 cells and Raji cells were collected (3 x 10^6 cells) in RPMI++, spun down (1500 rpm; 5 min), resuspended in 140 µl ^{51}Cr (Chromium-51; CJS11-1mCi, batch 12; 140 µl is about 100 µCi) and incubated

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(37 °C water bath; 1 hour). After washing cells (1500 rpm, 5 min, in PBS, 3x), cells were resuspended in RPMI++ and counted by trypan blue exclusion. Cells were brought at concentration of 2 x 10^4 cells/ml.

Preparation of effector cells: Fresh peripheral blood mononuclear cells (MNC) were isolated from 40 ml of heparin blood by Ficoll (Bio Whittaker; lymphocyte separation medium, cat 17-829E) via manufacturer's instructions. After resuspension of cells in RPMI++, cells were counted by trypan blue exclusion and adjusted to a concentration of 1 x 10^6 cells/ml.

ADCC set up: 50 µl RPMI++ was pipetted into 96 wells plates, and 50 µl of ^{51}Cr-labeled targets cells were added. Thereafter, 50 µl of antibody was added, diluted in RPMI++ (final concentrations 10, 1, 0.1, 0.01 µg/ml). Cells were incubated (RT, 10 min), and 50 µl effector cells were added, resulting in an effector to target ratio of 50:1 (for determination of maximal lysis, 50 µl 5% Triton-X-100 was added instead of effector cells). Cells were spun down (500 rpm, 5 min), and incubated (37 °C, 5% CO_2, 4 hours). After spinning down the cells (1500 rpm, 5 min), 100 µl of supernatant was harvested into micronic tubes, and counted in a gamma counter. The percentage specific lysis was calculated as follows:

% specific lysis = (cpm sample- cpm target cells only)/(cpm maximal lysis –cpm target cells only) x 100

Statistics: Data were analyzed by one-way ANOVA, followed by Tukey's multi comparison post-hoc test. Analysis was performed using Graph Pad Prism (version 3.02 for Windows, Graph Pad Software, San Diego, CA, USA).

Antibody concentration-dependent lysis of ARH-77and Raji cells: 2F2T and 11B8T were tested for their ability to induce ADCC of ARH-77 and Raji cells (n=3) in comparison with rituximab.

A dose-effect relation with CD20 mAbs was observed in ADCC of ARH-77 cells using MNC as effector cells (Figure 27). Both 2F2T and 11B8T induced specific lysis of ARH-77 cells which was maximal (50%) at 10 µg/ml of mAb. Rituximab induced only 25% lysis of target cells. Addition of the isotype control antibody (HuMab-KLH) did not induce ADCC. No specific lysis was observed without addition of MNCs (data not shown).

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When Raji cells were used as target cells, a similar picture as with ARH-77 cells emerged (Figure 28). Both 2F2T and 11B8T induced MNC-mediated lysis of Raji cells, albeit that 2F2T seemed more potent than 11B8T at low concentrations. The maximum lysis reached with 2F2T and 11B8T was approximately 35%. Rituximab induced MNC-mediated lysis of Raji cells, although only 20% of target cells were susceptible to rituximab. Addition of the isotype control antibody (HuMab-KLH) did not induce ADCC. No specific lysis was observed without addition of MNCs (data not shown).

Example 8 FRET and Triton-X insolubility analysis

Preparation of Cy3- and Cy5-conjugated mAb for fluorescence resonance energy transfer (FRET): Monoclonal antibodies were directly conjugated to bifunctional NHS-ester derivatives of Cy3 and Cy5 (Amserham Biosciences UK Ltd) as described in the manufacturer's instructions. Briefly, mAb were dialyzed against 0.1 M carbonate/bicarbonate buffer (pH 9). Thereafter, dye was dissolved in H_2O, immediately added to 1 mg of the mAb, and incubated at room temperature in the dark for 45 min. The labeled mAbs were separated from the unconjugated dye by gel chromatography using a PD10-Sephadex G25 column equilibrated in PBS. Molar ratios of coupling were determined spectrophotometrically from ε_{552} = 150/mM/cm for Cy3, ε_{650} = 250/mM/cm for Cy5, and ε_{280} = 170/mM/cm for protein, and ranged from 5- to 8-fold excess dye:protein.

FRET analysis: Daudi cells were resuspended at 5 x 10^6 cells/ml in PBS/0.1% BSA, and equimolar donor (Cy3)-conjugated and acceptor (Cy5)-conjugated mAb were combined and added to the cell suspension (final concentration 10 µg/ml). Cells were incubated for 30 min in the dark, at 4 °C or 37 °C. Each experiment included cells labeled with donor- and acceptor-conjugated mAb after pre-incubation with a 20-fold molar excess of unconjugated mAb, and cells labeled with donor-or acceptor-conjugated mAb in the presence of equimolar unlabeled mAb. To assess the association of labeled antigens, flow cytrometric FRET measurement was carried out using a FACScalibur (BD Biosciences). The fluorescence intensities at 585 nm (FL2) and 650 nm (FL3), both excited at 488 nm, and the fluorescence intensities at 661 nm (FL4), excited at 635 nm, were detected and used to calculate FRET according to the equitation below, where A is acceptor (Cy5), and D is donor (Cy3). All values obtained were corrected for autofluoresence using the following formula:

FRET = FL3(D,A)-FL2(D,A)/a-FL4(D,A)/b
where a = FL2(D)/FL3(D), and b = FL4(A)/FL3(A)

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Correction parameters were obtained using data collected from single-labeled cells, and side angle light scattering was used to gate out debris and dead cells. FRET between donor and acceptor mAb derivatives on dually labeled cells is expressed in terms of acceptor sensitized emission at 488 nm. Larger FRET values indicate closer physical association of the donor- and acceptor labeled antibodies or a higher density of acceptor-labeled mAb in the vicinity of donor-labeled mAb.

Assessment of raft associated antigen by Triton X-100 (TX) insolubility: As a rapid assessment of the presence of antigen in raft microdomains, a flow cytometry method based on Triton X-100 (TX) insolubility at low temperatures was used, as described previously. In brief, Daudi cells were washed in RPMI/ 1% BSA and resuspended at 2.5 x 10^6/ml. The cells (100 µl) were then incubated with 10 µg/ml of FITC conjugated mAb for 15 min at 37 °C, washed in cold PBS/ 1% BSA/ 20 mM sodium azide (PBS-BS), and the sample was divided in half. All samples were kept on ice throughout the remainder of the assay. One half was maintained on ice to allow calculation of 100% surface antigen levels, whilst the other was treated with 0.5% TX for 15 min on ice to determine to proportion of antigens remaining in the insoluble raft fraction. Cells were then maintained at 4 °C throughout the remainder of the assay, washed once in PBS-BS, resuspended in PBS-BS and assessed by flow cytometry. To determine the constitutive level of raft association of the target antigens, cells were first treated with 0.5% TX for 15 min on ice and washed in PBS-BS prior to binding of FITC-labeled mAb.

As shown in Figures 29A, 29B, and 29, fluorescence resonance energy transfer (FRET) analysis indicates clustering of CD20 upon incubation with 2F2 or 7D8. No such clustering was observed upon incubation with 11B8. These results are consistent with the TX treatment data, cf. Figure 29C, (*i.e.*, 2F2 and 7D8, unlike 11B8 remain with the insoluble fraction of the cell following binding) and support the concept that 2F2 and 7D8, upon binding, translocate CD20 into lipid raft compartment of the B cell membrane.

As shown if Figure 30 (FRET values and s.e.m. of three experiments using one-way ANOVA followed by Tukey's multi comparison post-hoc test) the FRET analysis indicates clustering of rituximab and 2F2, whereas no clustering was observed with 11B8T. These data are in agreement with the data obtained after treatment with 0.5% TX prior to binding of FITC-labeled mAbs as shown in Figure 31 (n=2).

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Preparation of lipid raft fractions and Western blotting: Another way to examine the association of CD20 with lipid rafts, is to investigate the distribution of CD20 between the raft and non-raft membrane fractions using the sucrose gradient fractionation method as disclosed by Deans, J.P., et al., *J. Biol. Chem.*, 1998. **273**(1): pp 344-348, except that Optiprep (Sigma) was used instead of sucrose. Monoclonal antibodies directed against CD20 (10 μg/ml) were allowed to bind to Daudi cells (1 x 10^7) for 20 min at 37 °C. Following this incubation, the cells were pelleted, washed twice with PBS and lysed in ice-cold lysis buffer (1.0% TX in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10 mM EDTA)). The cell pellet was resuspended thoroughly and incubated for 20 min on ice. Thereafter, the lysate was mixed with 400 μl cold 60% Optiprep (Sigma). The sample was overlaid with a 600 μl step of each 35%, 30%, 25%, 20%, 0% Optiprep in lysis buffer. The gradients were spun at 40.000 rpm at 4 °C for 18 hours. Six fractions from the top were collected, resolved on a 4-15% SDS-PAGE gel, transferred onto nitrocellulose membranes and incubated with primary antibody (mouse anti-CD20 polysera; Serotec, UK), followed by HRP-conjugated secondary antibody (rabbit anti mouse-HRP; Jackson, Bar Harbor, Maine, USA). Blots were visualised using Supersignal West Dura extended duration substrate (Pierce, Woburn, MA, USA).

The results are shown in Figure 32. As it can be seen, CD20 molecules are confined to the high-density fraction 5 (untreated cells). Cells treated with rituximab showed a distinct shift in CD20 distribution with a significant proportion in the lower density membrane fractions 2 and 3, coincident with the fraction where membrane rafts are expected to sediment. Cells treated with 2F2 also showed this shift to fractions 2 and 3. In contrast, cells treated with 11B8T for 20 min showed a similar distribution to untreated cells, with CD20 molecules in fraction 5. In conclusion binding to 2F2 and rituximab induces a shift of CD20 molecules to the lower density membrane fractions, whereas binding to 11B8T does not.

Example 9 Apoptosis of Burkitt Cell Lines with Human Antibodies Against CD20

Apoptosis: Daudi cells, 0.5 x 10^6 in 1 ml tissue culture medium, were placed into 24-well flat-bottom plates with 1 or 10 μg/ml mAb or control antibodies, and incubated at 37 °C. After 20 hours, cells were harvested, washed in Annexin-V-FITC binding buffer (BD biosciences) and labeled with Annexin V-FITC (BD biosciences) for 15 min in the dark at 4 °C. The cells were kept at 4 °C until analysis. Each sample (150 μl) was added to 10 μl of PI solution (10 μg/ml in PBS) in a FACS tube. The mixture was assessed immediately by flow cytometry using a FACScalibur flow cytometer with

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CellQuest pro software (BD Biosciences, Mountain view, CA). At least 10,000 events were collected for analysis.

Induction of apoptosis in Daudi cells: Daudi cells were incubated for 20 hours in the presence of human antibodies against CD20 (1 μg/ml) (without the addition of a secondary cross-linking antibody). Induction of apoptosis was assessed by AnnexinV/PI staining using flow cytometry.

As shown in Figures 33A-G, 11B8 shows clear evidence of inducing apoptosis (similar to that induced by an anti-IgM antibody). 2F2 and 7D8 did not induce apoptosis of Daudi cells. An apoptosis-inducing mouse anti-CD20 antibody, AT80, was used as a control.

Induction of apoptosis in Raji cells: Induction of apoptosis of Raji cells was tested with a concentration range of CD20 mAbs. Figure 34 shows the percentage of annexin-V-positive cells. As can be seen from Figure 34, the positive control mouse anti-human CD20-mAb, B1, induced a concentration-dependent increase in apoptosis of Raji cells with a maximum of approximately 70% at 10 μg/ml mAb. Also 11B8 was a strong inducer of apoptosis, resulting in apoptosis of Raji cells with a maximum of 53.4% at 10 μg/ml mAb. On the other hand, 2F2 and rituximab were very poor in inducing apoptosis of Raji cells, with slightly elevated levels of apoptosis compared to negative control levels.

Induction of apoptosis in Daudi cells: The same picture emerged when Daudi cells were used as target cells, after addition of 1.0 μg/ml CD20 mAb (Figures 35A and 35B). Data in Figure 35A show the total of annexin-V positive cells, and data in Figure 35B (the X-axis showing annexin-V, and the Y-axis showing PI) show the percentages of Daudi cells in early apoptosis (annexin-V positive and PI negative) and late apoptosis (annexin-V positive and PI positive). Again, Both B1 (65.9%) and 11B8T (56.3%) were strong inducers of apoptosis (Figure 36), when used at a concentration of 1.0 μg/ml. Addition of 2F2T resulted in a low level of apoptotic Daudi cells (17%). Addition of rituximab resulted in approximately 29% apoptosis of Daudi cells. Addition of isotype control antibody HuMab-KLH did not induce apoptosis of Daudi cells (6%).

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Example 10 Homotypic Adhesion of Cells with Human Antibodies Against CD20

Homotypic aggregation correlates with induction of apoptosis. Therefore, the ability of the anti-CD20 mAbs to induce homotypic aggregation of B cells was investigated

Homotypic aggregation of Ramos-EHRB cells: Ramos-EHRB cells (0.5 x 10^6 in 1 ml tissue culture medium) were incubated at 37 °C for 4 hours in the presence of anti-CD20 antibodies 11B8, 2F2, or 7D8 (without cross-linking) and induction of homotypic adhesion was assessed by light microscopy (as described above).

As shown in Figures 36A-E, 11B8 caused extensive aggregation of Ramos-EHRB cells (similar to the aggregation caused by murine anti-CD20 antibody, AT80). 2F2, and 7D8 did not induce homotypic aggregation of Ramos cells.

Homotypic aggregation of Daudi cells: Daudi cells were placed into 24-well flat-bottom plates with 1 or 10 µg/ml anti-CD20 mAbs or control antibody, and incubated at 37 °C for 4 hours. The extent of homotypic aggregation was determined by light microscopy. As can be seen from Figure 37, 2F2 hardly induced homotypic aggregation of Daudi cells, with 1.0 µg/ml (and 10 µg/ml, data not shown). Rituximab gave little homotypic aggregation of Daudi cells. In contrast, the B1 antibody was a strong inducer of homotypic aggregation.

Example 11 Immunotherapy Using Human Antibodies Against CD20

Therapy with high dose (100 µg) 2F2 and 7D8 of SCID mice challenged with Daudi cells: The SCID mice were obtained from Harlan UK Ltd., Blackthorn, Oxon, UK, and bred and maintained under pathogen free conditions. Daudi cells (2.5 x 10^6) were injected i.v. into the tail vein of cohorts of 12-16 weeks old SCID mice, followed 7 days later by injection of 100 µg of 2F2 or 7D8 via the same route. Animals were sacrificed upon presentation of limb paralysis, according to the instructions of the animal ethics committee. As shown in Figure 38, survival of the mice is prolonged after treatment with 2F2 or 7D8.

Therapy with high dose (100 µg) 2F2 and rituximab of SCID mice challenged with Tanoue cells: Tanoue cells (2.5 x 10^6 in 200 µl PBS) were injected i.v. into the tail vein of cohorts of 12-16 week old SCID mice (Harlan UK Ltd., Blackthorn, Oxon, UK) followed 7 days later by the injection of 100 µg (in 200 µl PBS) of anti-CD20 mAb via the same route. In this experiment, 2F2 was compared to rituximab and B1. Animals were sacrified upon presentation of rear-limb paralysis. The results are shown in Figure 39. At day 39, the first two control mice died, and death within this

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group was complete at day 54. Only one mouse died within this time interval following 2F2 treatment and survival was considerably increased for the other mice in this group. One mouse died 81 days following injection of the tumor cells and the remaining mice (60% of the total number) survived beyond the termination of the experiment at 100 days post tumor challenge. Rituximab in contrast only increased survival for 2 out of 5 mice (dying at 66 and 83 days post challenge) and none of the mice survived until the end of the experiment. In the B1-group, the survival of SCID mice was similar to that in the 2F2 group, with two mice dying on day 48, and one mouse on day 76. In this group, forty percent was alive at the time the experiment was terminated.

Dose response of 2F2 and rituximab treatment of SCID mice challenged with Daudi cells: To assess the efficacy of 2F2 in comparison to rituximab in protection against tumorigenesis, a dose titration was performed in therapy of SCID mice challenged with Daudi tumor cells. Daudi cells express more CD20 than Tanoue cells and are more sensitive to killing *in vitro*. 10 groups of SCID mice (4 per group) and 1 control group (5 SCID mice) were injected with 2.5 x 10^6 Daudi cells (in 200 µl PBS) i.v. on day 0, and then treated with 20, 5, 2, 0.5 or 0.1 µg (in 200 µl PBS) rituximab, 2F2 or PBS (control) i.v. on day 7. Animals were sacrificed upon presentation of rear-limb paralysis. The results are shown in Figure 40.

In the control group, all mice died within the time interval of 26-29 days. However, a clear dose-effect relation was observed with 2F2 (Figure 40, upper graph). Whereas no effect was observed with doses of 0.1 µg and 0.5 µg 2F2, as little as 2 µg 2F2 substantially extended survival until day 41, 5 µg 2F2 extended survival until day 47, and 20 µg 2F2 extended survival even until day 50.

In contrast, rituximab even tested at the highest dose of 20 µg only slightly increased survival and no dose-effect relation was therefore observed at the lower concentrations tested (Figure 40, lower graph).

Therapy of SCID mice with Daudi tumors by 11B8T and B1: Daudi cells (2.5 x 10^6) in 200 µl PBS were injected i.v. into the tail vein of cohorts of 12-16 week old SCID mice, followed 7 days later by the injection of 100 µg 11B8 or B1 in 200 µl PBS via the same route. Animals were sacrificed upon presentation of rear-limb paralysis. In control mice treated with PBS, all mice died within a time interval of 35-53 days (Figure 41). 11B8T treatment strongly protected the mice, with mice dying between 72 and 98 days post tumor challenge. In the B1-treatment group, most mice survived until day 98 and 40% of the mice survived beyond the end of the experiment, *i.e.*, day 100.

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Example 12 Evaluation of anti-CD20 antibodies in a Daudi-luc xenograft model using SCID mice

The therapeutic efficacy of anti-CD20 antibodies was evaluated in a mouse model in which disseminated outgrowth of human B-cell tumor cells is followed using external optical imaging. In this model tumor cells are transfected with firefly luciferase. Upon administration of luciferin (Molecular Probes, Leiden, The Netherlands) to the mice the labeled cells can be detected in *vivo* by bioluminescent imaging using a highly sensitive CCD camera, cf. Wetterwald *et al.* (2002) *American Journal of Pathology*, 160(3):1143-1153.

Daudi cells were transfected with gWIZ luciferase from Gene Therapy Systems (San Diego, CA) and cultured in RPMI with 10% FCS, Pen/Strep, Sodium Pyruvate and 1 ug/ml puromycin (Sigma). Cells were analysed for luciferase expression (expressed in RLU/1 x 10^5 cells) in a luminometer and for CD20 expression by FACS. 2.5 x 10^6 luciferase-transfected Daudi cells/mouse were injected i.v. into SCID mice. Eight days after inoculation, the mice received a single dose (10 μg) treatment of 2F2T, 11B8T, rituximab, B1 or isotype control antibody (huIgG1) (6 mice per treatment group). For imaging, mice were anesthetized by i.p. injection of a mixture of ketamine /xylazine /atropine. Synthetic D-Luciferin (sodium salt, Molecular Probes) was given i.p. at a dose of 25 mg/ml. Mice were then placed in a light tight box and after 3 min, imaging was started using a VersArray 1300B liquid nitrogen cooled CCD detector (Roper Scientific). Photons emitted from the luciferase were counted over an exposure period of 5 min. Under illumination black and white images were made for reference. MetaVue software (Universal Imaging Corp) was used for data collection and image analysis. Statistical significance of differences between groups was established using one-way analysis of variance with a Newman-Keuls post test using GraphPad PRISM version 3.02 (Graphpad Software Inc).

Imaging from the back side was performed at one-week intervals. On day 8, the day of treatment, light emission was only detected at the inoculation sites in the tail. Tumor formation at distant sites was detected on day 14 in all mice from the isotype control group (huIgG1) and in one mouse from the rituximab group. In the following weeks light emission steadily increased. Figure 42 gives the images of all mice made on day 39 (31 days after treatment), in which bioluminescence is represented in red color (the dark areas in the mice) (light intensity > 50 photons per 5 min) as overlay on the black and white body image of the mice. The tumor mass in each mouse was quantified on day 25, 32, 39, and 46 by integrating the light signals over the body surface, cf. Figure 43. The fastest tumor growth was observed in the isotype control group. Treatment with rituximab gave significant inhibition of tumor growth. However,

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tumor growth inhibition by 2F2T, 11B8T and B1 was significantly more potent (see below Table 2 for significance levels.

Table 2

Significance levels of differences in integrated light intensity between groups at different time points

	Day 25	Day32	Day 39	Day 46
B1 vs. rituximab	$P > 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.001$
B1 vs. 11B8T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
B1 vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
B1 vs. huIgG1	$P < 0.001$	$P < 0.001$	$P < 0.001$	
rituximab vs. 11B8T	$P > 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.001$
rituximab vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P < 0.001$
rituximab vs. huIgG1	$P < 0.001$	$P > 0.05$	$P < 0.05$	
11B8T vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
11B8T vs. huIgG1	$P < 0.001$	$P < 0.001$	$P < 0.001$	
2F2T vs. huIgG1	$P < 0.001$	$P < 0.01$	$P < 0.01$	

Example 13 Pilot and Pharmacokinetic study in cynomolgus monkeys

The objective was to determine the pharmacokinetic pattern and pharmacological effects of 2F2 in cynomolgus monkeys (approximately 2 years old; weight range of 2.1-2.6 kg) following once daily intravenous infusion administrations (*via* the saphenous vein) for 4 consecutive days. The study also compared the pharmacological effects of rituximab in order to determine its equivalent potential. For this purpose, 6 male and 6 female cynomolgus monkeys were assigned to 6 dose groups that received 2F2 or rituximab at dose levels of 1.25, 6.25 and 12.5 mg/kg/day at a constant dose volume of 10 ml/kg for 4 consecutive days, in total 5, 25 and 50 mg/kg, respectively. On completion of the last dose administration, the animals were retained for a post dose observation period of 130 days. The practices and procedures adopted during this study were consistent with the OECD Principles of Good Laboratory Practice as set forth by the United Kingdom Department of Health. All animals were observed at regular intervals for signs of ill health or reaction to treatment and were subjected to a physical examination. Laboratory investigations of haematology, coagulation, clinical chemistry and urine analysis were performed during the study. Blood samples and lymph node biopsies were obtained (from the superficial lymph nodes) for flow cytometry analysis throughout the dosing and post dose observation periods. The following cell phenotypes were analysed by flow cytometry: CD3, CD4, CD8, CD20

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and CD21. On completion of the post dose observation period the animals were sacrificed and subjected to a detailed necropsy.

There were no adverse clinical signs or any findings that were considered to be related to treatment with 2F2 or rituximab. Figures 44 and 45 shows the flow cytometry analysis of CD20 and CD21 expressing cells in peripheral blood of treated animals, respectively. Figure 46 shows the flow cytometry analysis of CD20 expressing cells in lymph nodes. Together, both phenotypes analysed during the study indicate a strong and efficient B cell depletion after administration of 2F2 and rituximab at 6.25 mg/kg/day (25 mg/kg in total) and 12.5 mg/kg/day (50 mg/kg in total). In addition, data shows that repopulation of CD20 expressing cells in the lymph nodes and peripheral blood of 2F2 treated animals restarted approximately at day 75 post dosing of 25 mg/kg and 50 mg/kg, *i.e.*, markedly later than in rituximab treated animals.

Furthermore, Figures 47A-C show the flow cytometric analysis of $CD20^{low}CD23^{+}CD40^{high}$ expressing cell subpopulations in the peripheral blood (Y. Vugmeyster *et al.* (2003) Cytometry 52A:101-109).

Peripheral blood cells obtained from either 2F2 or rituximab treated monkeys at dose levels of 1.25 mg/kg (Figure 47A), 6.25 mg/kg (Figure 47B), and 12.5 mg/kg (Figure 47C) once daily by intravenous infusion administrations for 4 consecutive days were incubated with anti-human CD20 FITC murine monoclonal antibody (Coulter) at room temperature for 10 min. Afterwards, count beads were added together with PBS and the cells were washed twice (300 g for 10 min), followed by immediate analysis of $CD20^{low}CD23^{+}CD40^{high}$ vs. $CD20^{high}$ $CD23^{+}CD40^{low}$ expressing cell subpopulation in a flow cytometer (Beckman Coulter). Results of $CD20^{low}$ $CD23^{+}CD40^{high}$ cells shown are expressed as cells per μl. As can be seen from the Figure 47 2F2 was capable of inducing a complete and longer depletion of $CD20^{low}CD23^{+}CD40^{high}$ expressing cells compared to rituximab.

Example 14 Epitope mapping using site-directed mutagenesis

Epitope mapping studies using a mutagenesis approach have indicated that alanine at position 170 (A170) and proline at position 172 (P172) in the second extracellular loop are critical for the recognition of human CD20 by known anti-CD20 antibodies. In studies by Deans and colleagues (M.J. Polyak, *et al.*, *Blood*, (2002) 99(9): pp 3256-3262; M.J. Polyak, *et al.*, *J. Immunol.*, (1998) 161(7): pp 3242-3248) the binding of all anti-CD20 mAbs tested was abrogated by changing A170 and P172 into the corresponding murine CD20 residues S170 and S172. Some heterogeneity in the recognition of the AxP epitope has been recognized however as most antibodies like rituximab recognize murine CD20 with S170 and S172 mutated to the human A170xP172 sequence whereas some others require additional mutations immediately N-

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terminal of the AxP sequence. To verify whether the A170xP172 motive is also important for the binding of the antibodies according to the invention the AxP sequence was mutated into SxS using site-directed mutagenesis (AxP mutant = A170S, P172S), cells were transfected with the AxP mutant and wild-type (WT) CD20 DNA, and the binding characteristics of the anti-CD20 mAbs were compared.

Further mutants were prepared, P172S (proline at position 172 mutated to serine), N166D (asparagine at position 166 mutated to aspartic acid), and N163D (asparagine at position 163 mutated to aspartic acid), using site-directed mutagenesis to evaluate whether the mutated amino acid residues are important for binding of the antibodies of the invention.

To examine this, a CD20 expression vector was constructed by amplifying the CD20 coding sequence using suitable primers introducing restriction sites and an ideal Kozak sequence for optimal expression. The amplified fragment was digested and ligated in the expression vector pEE13.4. After transformation in *E. coli*, colonies were screened for inserts and two clones were selected for sequencing to confirm the correct sequence. The construct was named pEE13.4CD20HS.

Mutagenesis was performed to introduce the AxP mutation and to introduce 20 mouse mutations in the extracellular loop regions of human CD20. Mutagenesis was checked by restriction enzyme digestion and sequencing. The constructs were transiently transfected in CHO cells (for AxP mutations) or HEK293F cells and analyzed 24 or 48 hours post-transfection using flow cytometry.

Oligonucleotide PCR Primers: Oligonucleotide primers were synthesized and quantified by Isogen BV (Maarssen, The Netherlands). Primers were reconstituted in water in a concentration of 100 pmol/µl and stored at -20 °C until required. A summary of PCR and sequencing primers is shown in Table 3.

Optical density determination of nucleic acids: Optical density was determined using an Ultrospec 2100 pro Classic (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The DNA concentration was measured by analysis of the OD_{260nm}, where one OD_{260nm} unit = 50 µg/ml. The reference solution was identical to the solution used to dissolve the nucleic acids.

Plasmid DNA isolation from E. coli culture: Plasmid DNA was isolated from *E. coli* cultures using kits from Qiagen according to the manufacturer's instructions (Westburg BV, Leusden, The Netherlands). For 'bulk' plasmid preparation either a Hi-Speed plasmid Maxi kit or a Hi-Speed plasmid Midi kit were used (Qiagen). For a small scale plasmid preparation (*i.e.*, 2 ml of *E coli* culture) a Qiaprep Spin Miniprep Kit

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(Qiagen) was used and the DNA eluted in 50 µl TE (Tris-HCl 10 mM pH 8.0, EDTA 1 mM).

PCR amplification: PCR reactions were performed according to the manufacturer's instructions for the Pfu-Turbo© Hotstart DNA polymerase (Stratagene, Amsterdam, The Netherlands). Each 20 µl reaction contained 1 x PCR reaction buffer, 200 µM mixed dNTPs, 6.7 pmol of each forward and reverse primer, approximately 1 ng template DNA and 1 unit of Pfu-Turbo© Hotstart DNA polymerase. PCR reactions were performed on a T-gradient Thermocycler 96 (Biometra GmbH, Goettingen, Germany) using a 30 cycle program of: +95 °C for 2 min, followed by 30 cycles of: +95 °C for 30 sec, anneal: a gradient of 45-65 °C for 30 sec and extension: +72 °C for 2 min, followed by a final extension step of 10 min at 72 °C and subsequent storage at 4 °C. The completed reactions were analysed by agarose gel electrophoresis.

Agarose gel electrophoresis: Agarose gel electrophoresis was performed according to Sambrook (Molecular Cloning Laboratory Manual, 3rd edition) using gels of 50 ml, in 1 x Tris/acetic acid/EDTA (TAE) buffer. DNA was visualized by the inclusion of ethidium bromide in the gel and observation under UV light. Gel images were recorded by a CCD camera and an image analysis system (GeneGnome; Syngene, Cambridge, UK).

Restriction enzyme digestions: Restriction enzymes were supplied by New England Biolabs (Beverly, MA) and used according to the supplier's recommendations. In general, 100 ng was digested with 5 units of enzyme(s) in appropriate buffer in a final volume of 10 µl. Reaction volumes were scaled up as appropriate. Digestions were incubated for a minimum of 60 min at the manufacturer's recommended temperature.

For fragments requiring double digestions with restriction enzymes which have incompatible buffer or temperature requirements, digestions were performed sequentially so as to offer favourable conditions for each enzyme in turn.

Alkaline phosphatase treatment: Shrimp alkaline phosphatase (USB, Cleveland, OH) was used according to the supplier's recommendations. Alkaline phosphatase removes 5'-phosphate groups from the ends of DNA fragments thereby preventing self-ligation. This is of particular relevance when self re-ligation of a DNA fragment could result in a replication-competent vector. The enzyme is active in most restriction enzyme buffers and was added as appropriate. After the digestion, the enzyme was inactivated by raising the temperature to 70 °C for 15 min.

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Purification of PCR and restriction enzyme reaction products: Purification was carried out using the mini-elute PCR Purification kit (supplied by Qiagen), according to the manufacturer's instructions. Briefly, DNA samples were diluted in 5 volumes of binding buffer I (Qiagen) and loaded onto a mini-elute column within an Eppendorf centrifuge tube. The assembly was centrifuged in a bench-top microcentrifuge. The column was washed twice with buffer II (Qiagen): Following buffer application, the assembly was centrifuged and the flow-through was discarded. The column was dried by centrifugation in the absence of added buffer. DNA was eluted by adding elution buffer to the column and the eluate collected by centrifugation. Isolated DNA was quantified by UV spectroscopy and quality assessed by agarose gel electrophoresis.

Isolation of DNA fragments from agarose gel: Where appropriate (*i.e.*, when multiple fragments were present), digested DNA samples were separated by gel electrophoresis and the desired fragment excised from the gel and recovered using the QIAEX II gel extraction kit (Qiagen), according to the manufacturer's instructions. Briefly, DNA bands were excised from the agarose gel and melted in an appropriate buffer at +55 °C. QIAEX II resin was added and incubated for 5 min. QIAEX II resin was pelleted by a short centrifugation step (1 min, 14000 g, RT) and washed twice with 500 µl of wash buffer PE. The final pellet was dried in a hood and DNA was eluted with the appropriate volume of TE and temperature (depending on the size of the DNA).

Ligation of DNA fragments: Ligations were performed with the Quick Ligation Kit (New England Biolabs) according to the manufacturer's instructions. For each ligation, the vector DNA was mixed with approximately 3-fold molar excess of insert DNA such that the total amount of DNA was lower than 200 ng in 10 µl, with volume adjusted with water as appropriate. To this was added 10 µl 2 x Quick Ligation Buffer and 1 µl Quick T4 DNA ligase and the ligation mix was incubated for 5-30 min at room temperature.

Transformation of DNA into bacteria: Samples of DNA were used to transform One Shot DH5α-T1R competent *E. coli* cells (Invitrogen, Breda, The Netherlands) using the heat-shock method according to the manufacturer's instructions. Briefly, 1-5 µl of DNA solution (typically 2 µl of DNA ligation mix) was added to an aliquot of transformation-competent bacterial cells and the mixture incubated on ice for 30 min. The cells were then heat-shocked by transferring to a waterbath at 42 °C for 30 sec followed by a further incubation on ice for 5 min. Cells were left to recover by

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incubation in a non-selective culture medium (SOC) for 1 hour with agitation at 37 °C and were subsequently spread onto agar plates containing appropriate selective agent (ampicillin at 50 µg/ml). Plates were incubated for 16-18 hours at +37 °C or until colonies of bacteria became evident.

Screening of bacterial colonies by PCR: Bacterial colonies were screened for the presence of vectors containing the desired sequences using the PCR colony screening technique. 20 µl of PCR reaction mix containing 0.5 volumes of HotStarTaq Master Mix (Qiagen), 4 pmol of the forward and reverse primers and completed with water was added to a PCR tube. A colony was lightly touched with a 20 µl pipet tip, once touched in 2 ml LB in a culture tube (for growing bacteria containing the corresponding plasmid) and resuspended in the 20 µl PCR mix. PCR was performed on a T-gradient Thermocycler 96 (Biometra) using a 35 cycle program of: +95 °C for 15 min, followed by 35 cycles of: +94 °C for 30 sec, anneal: 55 °C for 30 sec and extension: +72 °C for 2 min, followed by a final extension step of 10 min at 72 °C and subsequent storage at 4°C. The completed reactions were analyzed by agarose gel electrophoresis. See Table 3 for details of primer pairs used for colony PCR.

DNA sequencing: Plasmid DNA samples were send to AGOWA (Berlin, Germany) for sequence analysis. Sequences were analyzed using the VectorNTI software package (Informax, Frederick, MD, USA).

Table 3

Name	Application	Length	Oligo Sequence
CD20P172S	CD20 mutagenesis	36	TGGGGAGTTTTTCTCAGAGGAATTCGATGGTTCACAGTTGTA
CD20N166D	CD20 mutagenesis	39	TGTAACAGTATTGGGTAGATGGG
CD20N163D	CD20 mutagenesis	36	AATCATGGACATACTTAATATTA
cd20exfor	CD20 construction	41	TATAGCCCGGGGCCGCCACCATGACAACACCCAGAAATTCA
cd20exrev	CD20 construction	38	GCGTCTCATGTACATTAAGGAGAGCTGTCATTTTCTAT
pee13.4seqrev2	Colony PCR	23	TCGGACATCTCATGACTTTCTTT
pConKseq1	Colony PCR	23	GTAGTCTGAGCAGTACTCGTTGC
cd20hsapmutr (AxP)	CD20 mutagenesis	42	TGGGGAGTTTTTCTCAGAGGAATTCGATGGTTCACAGTTGTA
cd20hsapmutf (AxP)	CD20 mutagenesis	42	TACAACTGTGAACCATCGAATTCCTCTGAGAAAAACTCCCCA
CD20seq2	CD20 sequencing	23	TGTAACAGTATTGGGTAGATGGG
cd20seq1	CD20 sequencing	23	AATCATGGACATACTTAATATTA

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Mutagenesis: The mutagenesis was performed, using either the QuikChange® XL Site-Directed Mutagenesis kit (Cat 200517-5, Lot 1120630, Stratagene Europe) according to the manufacturer's instructions.

Mutagenesis reactions were concentrated using ethanol precipitation and transformed into either oneshot DH5α-T1R competent *E. coli* cells or electroporated into ElectroTen-Blue® Electroporation-Competent Cells. Colonies were checked by colony PCR and restriction digestion prior to transfection.

HEK293F cell transfection: HEK293F cells were obtained from Invitrogen and transfected according to the manufacturer's instructions, using 293fectin. The HEK293F cells were used for all the single mutant sequences.

CHO cell transfection: CHO cells grown to approximately 95% confluence were transiently transfected with CD20 wild-type, mutant cDNA or a combination of both constructs using lipofectamine 2000 (M668-019, Invitrogen, Breda, Netherlands). To this end, 24 µg precipitated DNA was diluted (1µg/µl) in 500 µl optimem, in ratios of AxP 100% : WT 0%; AxP 33.3% : WT 66.6%; AxP 66.6% : WT 33.3%;AxP 0% :WT 100%. For each transfection 24 µl lipofectamine was diluted in 500 µl optimem. Then, the diluted lipofectamine was incubated (RT, 5 min), and the diluted DNA combined with the diluted lipofectamine. After gently mixing and incubating the solution (RT, 20 min), 1000 µl DNA/lipofectamine was added to the CHO cells, thoroughly mixed and incubated for 48 hours at 37 °C, 5% CO_2. Two days after transfection of CHO cells, cells were washed twice with FACS buffer (PBS supplemented with 0.1% BSA and 0.002% NaN_3). CHO cells were treated with trypsin/EDTA (Gibco BRL, Life Technologies, Paisley, Scotland) and lifted off the culture plates.

Anti-CD20 Antibody binding: HEK293F cells and CHO cells were taken up in PBS in a concentration of 2 x 10^6/ml, and added to round bottom plates (1 x 10^5/well). Then, 50 µl CD20 mAb was added, in serial dilutions of 10, 5, 2.5, or 0 µg per well (4 °C, 30 min). After washing in FACS buffer (PBS supplemented with 0.1% BSA and 0.002% NaN_3), the cells were analyzed on a flow cytometer (Becton Dickinson, San Diego, CA, USA), and 5,000 events per sample were acquired at high flow rate.

As can be seen from Figures 48A-E, all anti-CD20 mAbs bound efficiently to CHO cells expressing WT CD20. As expected, rituximab did not bind the AxP mutant (Figure 48A), and B1 bound this mutant poorly (Figure 48D). Both 2F2 and 11B8 in contrast bound to WT and AxP mutant CD20 equally well (Figure 48B and

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Figure 48C). Titrating the amount of WT CD20 on the surface indeed titrated the binding of rituximab and B1. Both 2F2 and 11B8 again were insensitive to the absence or presence of the mutation.

This study indicates that the binding of 2F2 and 11B8 to human CD20 is insensitive to mutations at amino acid positions 170 and 172. 2F2 and 11B8 therefore represent a new class of CD20 mAbs recognizing a novel CD20 epitope.

Figure 49A shows percentage binding of 2F2, 11B8T, B1 or rituximab to mutant P172S vs. WT CD20, Figure 49B shows percentage binding of 2F2T, 11B8T, B1, CAT (CAT 13.6E12, a mouse monoclonal IgG2A anti-CD20 antibody, Diatec.Com), a control isotype antibody (KLH), or rituximab to mutant CD20 (AxP) vs. WT CD20.

For the mutant wherein asparagine at position 166 has been replaced with aspartic acid (CD20N166D) 2F2 showed very low binding, whereas B1, rituximab and 11B8T were able to bind, see Figure 49C. In a similar experiment CAT 13.6E12 and rituximab were able to bind to CD20N166D, whereas 2F2T only showed very low binding, see Figure 49D. For the mutant wherein asparagine at position 163 has been replaced by aspartic acid (CD20N163D) again rituximab, 11B8T, and B1 were able to bind to CD20N163D, whereas 2F2 and 2F2T only showed very low binding, see Figure 49E. In a similar experiment CAT 13.6E12 and rituximab were able to bind to CD20N163D, whereas 2F2T only showed very low binding, see Figure 49F.

These experiments indicate that 2F2 and 11B8 bind to different epitopes.

Example 15 Epitope mapping using Pepscan method

Synthesis of peptides: 7-, 9-, and 15-mer peptides were synthesized according to standard methods. In some cases chemical linkage of the legs of a 15-mer peptide helps to identify amino acid sequences of a potentially discontinuous epitope. According to known procedures (H.M. Geysen *et al.* (1984) *Proc. Natl. Acad. Sci. USA,* 81:3998; J.W. Slootstra *et al.* (1996) *Mol. Divers.* 1:87; and WO 01/60769), 7-, 9-, and 15-mer peptides were synthesized that could be possible binding sites or epitopes involved in binding of 2F2 or 11B8 to the human CD20 molecule. The 9- and 15-mers were synthesized as loops and screened using credit-card format mini-PEPSCAN cards (455 peptide format/card). In all looped peptides amino acids at varied positions were replaced by a cysteine (*e.g.*, acetyl-XCXXXXXXXXXXXXCX-minicard). The peptides were synthesized using standard Fmoc-chemistry and deprotected using TFA with scavengers. Subsequently, the deprotected peptides were reacted on the microarray with an 0.5 mM solution of 1,3-bis(bromomethyl)benzene in ammonium bicarbonate (20 mM, pH 7.9), supplemented with acetonitrile (1:1 (v/v)). The microarrays were gently shaken in the solution for 30-60 min, while completely covered in the solution. Finally,

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the microarrays were washed extensively with excess of Millipore H_2O and sonicated in disrupt-buffer containing 1% sodium dodecylsulfate, 0.1% β-mercaptoethanol, in PBS (pH 7.2) at 70 °C for 30 min, followed by sonication in millipore H_2O for another 45 min. Subsequently, the microwells were ready for screening in an ELISA-assay.

Pepscan ELISA-assay: The 455-well credit card-format polyethylene cards, containing the covalently linked peptides, were incubated with serum (diluted 1:1000 in blocking solution which contains 5% horse serum (v/v) and 5% ovalbumin (w/v)) (4 °C, over night). After washing, the peptides were incubated with anti-human antibody peroxidase (dilution 1:1000, 1 hour, 25 °C), and after washing the peroxidase substrate, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate and 2 µl/ml 3% H_2O_2 were added. After one hour, the color development was measured. The color development of the ELISA was quantified with a CCD-camera and an image processing system. The set up consists of a CCD-camera and a 55 mm lens (Sony CCD Video Camera XC-77RR, Nikon micro-nikk or 55 mm f/2.8 lens), a camera adaptor (Sony Camera adaptor DC-77RR) and the Image Processing Software package Optimas, version 6.5 (Media Cybernetics, Silver Spring, MD 20910, U.S.A.). Optimas runs on a pentium II computer system.

The absorbances (OD values) for the peptides at different antibody concentrations are shown in below Table 4 and Table 5.

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GMI-055PC

Table 4

	11B8 10 μg/ml	11B8 100 μg/ml	7D8 10 μg/ml	7D8 100 μg/ml	rituximab 10 μg/ml	2F2 10 μg/ml	2F2 100 μg/ml	B1 10 μg/ml	B1 100 μg/ml
KMECLNFIRAHCPYI	**763**	**2997**	134	41	90	48	66	147	304
LKMECLNFIRCHTPY	165	738	160	41	120	49	87	179	216
KMESCNFIRACTPYI	**625**	**3090**	142	52	123	39	78	170	308
MESLCFIRAHCPYIN	179	956	127	55	102	41	65	119	178
CFIRAHTPC	188	534	181	69	134	91	114	170	212
CIRAHTPYC	151	449	186	60	132	57	92	151	195
CRAHTPYIC	**427**	**1605**	188	64	145	48	87	179	216
CAHTPYINC	179	452	174	65	125	42	106	161	172
IPAGIYA	217	950	164	76	177	48	85	165	192
PAGIYAP	**449**	**2501**	170	64	111	43	85	165	300
AGIYAPI	**251**	**2207**	188	73	110	44	98	187	143
GIYAPIC	99	251	152	64	141	34	93	177	147
IYAPICV	137	313	174	58	159	58	99	175	90
GIYAPIA	172	857	177	96	156	62	96	165	121
IYAPIAV	161	654	181	58	116	62	76	161	106

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Table 5

	11B8 10 μg/ml	7D8 10 μg/ml	rituximab 10 μg/ml	2F2 10 μg/ml
PCINIYNAEPANPCE	118	163	152	65
YCNIYNAEPANPSCK	287	181	**2418**	86
ICIYNAEPANPSECN	138	192	142	78
NCYNAEPANPSEKCS	93	121	**2649**	49
ICNAEPANPSEKNCP	115	165	**3283**	43
YCAEPANPSEKNSCS	106	188	**3770**	65
NCEPANPSEKNSPCT	159	183	**3476**	61
ACPANPSEKNSPSCQ	146	148	**250**	77
ECANPSEKNSPSTCY	134	179	188	68

As appears from Table 4, 11B8 showed binding to AGIYAP of the small first extracellular loop of human CD20 at both 10 μg/ml and 100 μg/ml, whereas the other antibodies tested did not show significant binding to AGIYAP.

Furthermore, 11B8 showed binding to MESLNFIRAHTPYI of the second extracellular loop of human CD20 at both 10 μg/ml and 100 μg/ml, whereas the other antibodies tested did not show significant binding to MESLNFIRAHTPYI.

As appears from Table 5, rituximab showed binding to EPANPSEK of the second extracellular loop of human CD20 at both **1** μg/ml and 10 μg/ml, whereas the other antibodies tested did not show significant binding to EPANPSEK.

Example 16 Anti-idiotypic antibodies

Generation of anti-idiotypic antibodies: Mouse anti-idiotypic antibodies were made by immunizing Balb/C mice with 2F2 or 11B8T, and generating hybridomas from spleens of these mice by fusion with NS1 myeloma cells using standard techniques. The following anti-idiotypic antibodies were generated: anti-2F2 sab 1.1, anti-2F2 sab 1.2, anti-2F2 sab 1.3, anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6. These were tested for specific binding to 2F2T, 7D8 and 11B8T. ELISA plates were coated with purified 2F2T, 7D8 or 11B8T (diluted in PBS to a final concentration of 1-2 μg/ml, 37 °C, 2 hours). Plates were blocked with PBS containing 0.05% Tween-20 and 2% chicken serum (RT, 1 hour). Subsequently, the plates were incubated with supernatants from cultures of the anti-idiotypic antibodies (final concentration adjusted to 1-10 μg/ml, RT, 2 hours). Bound mouse anti-idiotypic antibodies were detected with rabbit-anti-mouse IgG-HRP conjugated antibody (Jackson ImmunoResearch).

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As shown in Figure 50 anti-2F2 sab 1.1, anti-2F2 sab 1.2, and anti-2F2 sab 1.3 bind to 2F2T and 7D8, but not to 11B8T or an unrelated, isotype control human antibody. Since 2F2T and 7D8 are very homologous in V_L and V_H sequence, reaction of anti-2F2 idiotypic antibodies with 7D8 was expected.

Figure 51 shows that anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6 all bind to 11B8T to a similar extent.

Anti-idiotypic antibodies as an immunodiagnostic tool: The 2F2/7D8 and 11B8T specific anti-idiotypic antibodies can be used as an immunodiagnostic tool to detect and quantify levels of human monoclonal antibodies against CD20 in laboratory or patient samples. This may be useful for examining pharmakokinetics of the anti-CD20 antibody or for determining and adjusting the dosage of the anti-CD20 antibody and for monitoring the disease and the effect of treatment in a patient. As an example of such an assay, ELISA plates were coated with 4 μg/ml anti-2F2 sab 1.1, anti-2F2 sab 1.2 or anti-2F2 sab 1.3. Plates were blocked with PBS containing 0.05% Tween-20 and 2% chicken serum (RT, 1 hour). Subsequently, the plates were incubated with a serial dilution of 2F2T (10,000-9.77 ng/ml, RT, 2 hours). Bound 2F2T was detected with mouse-anti-human IgG HRP-conjugated antibody. As shown in Figures 52A-C a dose dependent binding of 2F2T was observed.

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Any combination of the embodiments disclosed in the dependent claims are also contemplated to be within the scope of the invention.

Incorporation by Reference

All patents, pending patent applications and other publications cited herein are hereby incorporated by reference in their entirety.

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We claim:

1. An isolated human monoclonal antibody which binds to human CD20.

2. The antibody of claim 1, selected from the group consisting of an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, a secretory IgA, an IgD, and an IgE antibody.

3. The antibody of claim 2, wherein the antibody is an IgG1 antibody.

4. The antibody of claim 2, wherein the antibody is an IgG3 antibody.

5. The antibody of claim 2, wherein the antibody is an IgG4 antibody.

6. The antibody of claim 2, wherein the antibody is an IgA1 or IgA2 antibody.

7. The antibody of any one of the preceding claims, wherein the antibody dissociates from human CD20 with a dissociation rate constant (k_d) of about 10^{-5} sec^{-1} or less.

8. The antibody of any one of the preceding claims, wherein the antibody binds to human CD20 with an affinity constant (K_D) of about 5 nM or less.

9. The antibody of any one of the preceding claims, wherein the antibody has one or more of the characteristics selected from the group consisting of:

(i) capable of inducing complement dependent cytotoxicity (CDC) of cells expressing CD20 in the presence of complement;

(ii) capable of inducing complement dependent cytotoxicity (CDC) of cells expressing CD20 and high levels of CD55 and/or CD59 in the presence of complement;

(iii) capable of inducing apoptosis of cells expressing CD20;

(iv) capable of inducing antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20 in the presence of effector cells;

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(v) capable of inducing homotypic adhesion of cells which express CD20;

(vi) capable of translocating into lipid rafts upon binding to CD20;

(vii) capable of prolonging the survival of a subject having tumor cells which express CD20;

(viii) capable of depleting cells expressing CD20; and

(ix) capable of depleting cells expressing low levels of CD20 ($CD20^{low}$ cells).

10. The antibody of claim 9, which has one or more of the following characteristics selected from the group consisting of:

(i) capable of inducing at least 20%, and preferably at least 30% CDC mediated lysis of B-CLL cells in the presence of 33 vol/vol% plasma within 3 hours at 37 °C at an antibody concentration of 10 μg/ml;

(ii) capable of inducing at least 20%, and preferably at least 30% lysis of B-CLL cells in the presence of 33 vol/vol% whole blood cells within 3 hours at 37 °C at an antibody concentration of 10 μg/ml;

(iii) capable of prolonging the 50% survival rate of SCID mice injected with Daudi cells by more than 30% at a dose of 20 μg; and

(iv) capable of depleting peripheral B cells expressing low levels of CD20 ($CD20^{low}$ B cells) to undetectable levels for more than 50 days in cynomolgus monkeys at a dosage of 6.25 mg/kg per day for 4 consecutive days.

11. The antibody of claim 10, which is capable of capable of inducing at least 20%, and preferably at least 30% CDC mediated lysis of B-CLL cells in the presence of 33 vol/vol% plasma within 3 hours at 37 °C at an anti-CD20 antibody concentration of 10 μg/ml.

12. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:1 and SEQ ID NO:3, respectively, and conservative sequence modifications thereof.

13. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:5 and SEQ ID NO:7, respectively, and conservative sequence modifications thereof.

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14. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:9 and SEQ ID NO:11, respectively, and conservative sequence modifications thereof.

15. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively, and conservative sequence modifications thereof.

16. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively.

17. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:6 and SEQ ID NO:8, respectively, and conservative sequence modifications thereof.

18. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:6 and SEQ ID NO:8, respectively.

19. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:10 and SEQ ID NO:12, respectively, and conservative sequence modifications thereof.

20. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:10 and SEQ ID NO:12, respectively.

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21. The antibody of any of the preceding claims 1-11 comprising at least one human variable region selected from the group consisting of:

(i) SEQ ID NOs:2, 4, 6, 8, 10, or 12; and

(ii) a sequence which is at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to any one of the amino acid sequences as set forth in (i) above.

22. An isolated human monoclonal antibody which binds to an epitope on human CD20 defined by the antibody of any one of claims 12-21.

23. An isolated human monoclonal antibody which binds to an epitope on CD20

(i) which does not comprise or require the amino acid residue proline at position 172;

(ii) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172;

(iii) which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166;

(iv) which does not comprise or require the amino acid residue proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; or

(v) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166.

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24. An isolated human monoclonal antibody which binds to CD20, wherein the antibody has one or more of the following characteristics:

(i) binds to mutant P172S CD20 (proline at position 172 mutated to serine) with at least the same affinity as to human CD20;

(ii) binds to mutant AxP (alanine at position 170 mutated to serine, and proline at position 172 mutated to serine) with at least the same affinity as to human CD20;

(iii) shows a reduced binding of 50% or more to mutant N166D (asparagine at position 166 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml; or

(iv) shows a reduced binding of 50% or more to mutant N163D (asparagine at position 163 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml.

25. An isolated human monoclonal antibody which binds to an epitope in the small first extracellular loop of human CD20.

26. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20.

27. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20, wherein the epitope comprises part of the first small extracellular loop and part of the second extracellular loop.

28. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20, wherein the epitope has residues AGIYAP of the small first extracellular loop and residues MESLNFIRAHTPYI of the second extracellular loop.

29. An isolated human monoclonal antibody which has the binding characteristics of the antibody of any one of claims 11-28.

30. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 13, 14, 15, 16, 17, or 18;

(ii) conservative sequence modifications of the sequences listed in (i); and

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(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

31. The antibody of claim 30, comprising SEQ ID NO:15.

32. The antibody of claim 30, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 13, 14, 15, 16, 17, or 18;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

33. The antibody of claim 30, comprising SEQ ID NOs: 13, 14, 15, 16, 17, and 18.

34. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 19, 20, 21, 22, 23, or 24;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

35. The antibody of claim 34, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 19, 20, 21, 22, 23, or 24;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

36. The antibody of claim 34, comprising SEQ ID NOs: 19, 20, 21, 22, 23, and 24.

37. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 25, 26, 27, 28, 29, or 30;

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(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

38. The antibody of claim 37, comprising SEQ ID NO:27.

39. The antibody of claim 37, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 25, 26, 27, 28, 29, or 30;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

40. The antibody of claim 39, comprising SEQ ID NOs: 25, 26, 27, 28, 29, and 30.

41. The antibody of any of the preceding claims 1-11 comprising at least one CDR selected from the group consisting of

(i) SEQ ID NOs:13, 19, or 25, or a sequence having 1 amino acid substitution, deletion or addition of the sequence of SEQ ID NOs:13, 19 or 25;

(ii) SEQ ID NOs:14, 20, or 26, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:14, 20, or 26;

(iii) SEQ ID NOs:15, or 27, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27;

(iv) SEQ ID NO: 16, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:16;

(v) SEQ ID NO: 17, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:17; and

(vi) SEQ ID NOs: 18, or 30, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs: 18, or 30.

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42. The antibody of claim 41 comprising at least four CDRs selected from the group consisting of

(i) SEQ ID NOs:13, 19, or 25, or a sequence having 1 amino acid substitution, deletion or addition of the sequence of SEQ ID NOs:13, 19 or 25;

(ii) SEQ ID NOs:14, 20, or 26, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:14, 20, or 26;

(iii) SEQ ID NOs:15, or 27, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27;

(iv) SEQ ID NO: 16, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:16;

(v) SEQ ID NO: 17, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:17; and

(vi) SEQ ID NOs: 18, or 30, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs: 18, or 30.

43. The antibody of claim 41 comprising one CDR selected from the group consisting of

SEQ ID NOs:15, or 27, and a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27.

44. The antibody of any one of the preceding claims which is an intact antibody selected from the group consisting of: an intact IgG1 antibody, an intact IgG2 antibody, an intact IgG3 antibody, an intact IgG4 antibody, an intact IgM antibody, an intact IgA1 antibody, an intact IgA2 antibody, an intact secretory IgA antibody, an intact IgD antibody, and an intact IgE antibody, wherein the antibody is glycosylated in a eukaryotic cell.

45. The antibody of any one of the preceding claims which is an antibody fragment or a single chain antibody.

46. The antibody of any one of the preceding claims which is a binding-domain immunoglobulin fusion protein comprising (i) a binding domain polypeptide in the form of a heavy chain variable region or a light chain variable region as defined in claim 24 that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region.

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47. The antibody of any one of the preceding claims produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, in which V-(D)-J gene segment rearrangements have resulted in the formation of a functional human heavy chain transgene and a functional human light chain transgene, fused to an immortalized cell.

48. A hybridoma comprising a B cell obtained from a transgenic non-human animal in which V-(D)-J gene segment rearrangements have resulted in the formation of a functional human heavy chain transgene and a functional light chain transgene fused to an immortalized cell, wherein the hybridoma produces a detectable amount of the monoclonal antibody of any one of the preceding claims.

49. A hybridoma which produces a human monoclonal antibody encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof.

50. A hybridoma which produces a human monoclonal having IgG heavy chain and kappa light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs: 4, 8, or 12, respectively, and conservative sequence modifications thereof.

51. The antibody of any one of claims 1-45 produced by a transfectoma comprising nucleic acids encoding a human heavy chain and a human light chain.

52. A transfectoma comprising nucleic acids encoding a human heavy chain and a human light chain, wherein the transfectoma produces a detectable amount of the antibody of any one of claims 1-45.

53. A transfectoma which produces a human monoclonal antibody encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof.

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54. A transfectoma which produces a human monoclonal antibody having IgG heavy chain and kappa light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

55. A eukaryotic or prokaryotic host cell which produces a human monoclonal antibody having heavy chain and light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

56. A transgenic non-human animal or plant which produces a human monoclonal antibody having heavy chain and light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

57. A method of producing a human monoclonal antibody which binds to human CD20, comprising:

immunizing a transgenic non-human animal having a genome comprising a human heavy chain transgene and a human light chain transgene with human CD20 or a cell expressing human CD20, such that antibodies are produced by B cells of the animal;

isolating B cells of the animal;

fusing the B cells with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies specific for human CD20; and

isolating the human monoclonal antibodies specific for CD20 from the culture supernatant of the hybridoma, or the transfectoma derived from such hybridoma.

58. A method according to claim 57, wherein the immunization is performed with cells which have been transfected with human CD20.

59. An isolated human monoclonal antibody which binds to human CD20 obtained by:

immunizing a transgenic non-human animal having a genome comprising a human heavy chain transgene and a human light chain transgene with a cell which has been transfected with human CD20, such that antibodies are produced by B cells of the animal;

isolating B cells of the animal;

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fusing the B cells with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies specific for human CD20; and

isolating the human monoclonal antibodies specific for CD20 from the culture supernatant of the hybridoma, or the transfectoma derived from such hybridoma.

60. An isolated human antibody comprising a heavy chain variable region amino acid sequence derived from a human V_H3-13/DP-44 germline sequence (SEQ ID NO:54) and a light chain variable region amino acid sequence derived from a human L6/JK4-CK (SEQ ID NO:55) germline sequence, wherein the human antibody binds to human CD20.

61. An isolated human antibody comprising a heavy chain variable region amino acid sequence derived from a human V_H3-09/JH6b germline sequence (SEQ ID NO:56) and a light chain variable region amino acid sequence derived from a human V_L-L6/JK5 germline sequence (SEQ ID NO:57), wherein the human antibody binds to human CD20.

62. A composition comprising the human antibody of any one of claims 1-45 and a pharmaceutically acceptable carrier.

63. A composition comprising a combination of two or more human antibodies according to any one of claims 1-45, which have complementary functional activities.

64. A composition according to claim 61 comprising a first antibody according to claim 15 and a second antibody according to claim 19.

65. A composition according to any of claims 62-64 further comprising a therapeutic agent.

66. The antibody according to any one of claims 1-45, further comprising a chelator linker for attaching a radioisotope.

67. An immunoconjugate comprising an antibody according to any one of claims 1-45 linked to a cytotoxic agent, a radioisotope, or a drug.

68. A bispecific molecule comprising an antibody according to any one of claims 1-45 and a binding specificity for a human effector cell.

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69. A bispecific molecule comprising an antibody according to any one of claims 1-45 and a binding specificity for a human Fc receptor or a binding specificity for a T cell receptor, such as CD3.

70. A method of inhibiting growth of a cell expressing CD20, comprising contacting the cell with an effective amount of an antibody according to any one of claims 1-45 such that the growth of the cell is inhibited.

71. A method of killing a cell expressing CD20, comprising contacting the cell with the antibody of any one of claims 1-45, such that killing of the cell expressing CD20 occurs.

72. The method of any one of claims 70 or 71, wherein the cell is a B lymphocyte or a tumor cell.

73. A method of treating or preventing a disease or disorder involving cells expressing CD20, comprising administering to a subject a human antibody, composition, immunoconjugate, or bispecific molecule according to any one of claims 1-45 or 60-69, an expression vector according to any one of the claims 94-97, in an amount effective to treat or prevent the disease.

74. The method of claim 73, wherein the disease is a B cell lymphoma.

75. The method of claim 73, wherein the disease is B cell non-Hodgkin's lymphoma.

76. The method of claim 73, wherein the disease is selected from the group consisting of precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL).

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77. The method of claim 76, wherein the disease is follicular lymphoma (FL).

78. The method of claim 76, wherein the disease is B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL).

79. The method of claim 73, wherein the disease is selected from the group consisting of lymphomatoid granulomatosis, primary effusion lymphoma, intravascular large B cell lymphoma, mediastinal large B cell lymphoma, heavy chain diseases (including γ, μ, and α disease), lymphomas induced by therapy with immunosuppressive agents, such as cyclosporine-induced lymphoma, and methotrexate-induced lymphoma.

80. A method of treating or preventing an immune disease involving CD20 expressing immune cells, comprising administering to a subject the antibody, composition, immunoconjugate, or bispecific molecule according to any one of claims 1-45 or 60-67 or an expression vector according to any one of the claims 94-97, in an amount effective to treat or prevent the immune disease.

81. The method of claim 80, wherein treatment includes the killing of B cells which produce antibodies against autoantigens.

82. The method of claim 73, wherein the disease or disorder is selected from the group consisting of psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases.

83. The method of claim 82, wherein the autoimmune disease is rheumatoid arthritis (RA).

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84. The method of claim 73, wherein the disease is an inflammatory, immune and/or autoimmune disorder selected from ulcerative colitis, Crohn's disease, juvenile onset diabetes, multiple sclerosis, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, systemic sclerosis, and pemphigus vulgaris.

85. The method of claim 73, wherein the disease is an inflammatory, immune and/or autoimmune disorder selected from inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, and multiple sclerosis.

86. The method of any one of claims 70-85, further comprising separately administering another therapeutic agent to the subject.

87. The method of claim 86, wherein the therapeutic agent is a cytotoxic agent or a radiotoxic agent.

88. The method of claim 86, wherein the therapeutic agent is an immunosuppressant.

89. The method of claim 86, wherein the therapeutic agent is an immunological modulating agent, such as a cytokine or a chemokine.

90. The method of claim 86, wherein the therapeutic agent is selected from the group consisting of doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide.

91. The method of claim 86, wherein the therapeutic agent is selected from the group consisting of anti-CD25 antibodies, anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, anti-CD38 antibodies, anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies, anti-alpha-4/beta-1 integrin (VLA4) antibodies, CTLA4-Ig, and anti-C3b(i) antibodies.

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92. An *in vitro* method for detecting the presence of CD20 antigen, or a cell expressing CD20, in a sample comprising:

contacting the sample with the antibody of any one of claims 1-45 under conditions that allow for formation of a complex between the antibody and CD20; and

detecting the formation of a complex.

93. A kit for detecting the presence of CD20 antigen , or a cell expressing CD20, in a sample comprising the antibody of any one of claims 1-45.

94. An *in vivo* method for detecting CD20 antigen, or a cell expressing CD20, in an subject comprising:

administering the antibody of any one of claims 1-45 under conditions that allow for formation of a complex between the antibody and CD20; and

detecting the formed complex.

95. An expression vector comprising a nucleotide sequence encoding the variable region of a light chain, heavy chain or both light and heavy chains of a human antibody which binds human CD20.

96. The expression vector of claim 95, further comprising a nucleotide sequence encoding the constant region of a light chain, heavy chain or both light and heavy chains of a human antibody which binds human CD20.

97. An expression vector comprising a nucleotide sequence encoding a heavy chain variable region comprising a nucleotide selected from the group consisting of the nucleotide sequences as set forth in SEQ ID NOs: 1, 5, and 9, and a light variable region comprising a nucleotide sequence selected from the group consisting of the nucleotide sequences as set forth in SEQ ID NOs: 3, 7, and 11, and conservative modifications thereof.

98. An expression vector comprising a nucleotide sequence encoding a heavy chain variable region comprising an amino acid sequence selected from the group consisting of the amino acid sequences as set forth in SEQ ID NOs:2, 6, and 10, and a light chain variable region comprising the amino acid sequence selected from the group consisting of the amino acid sequences as set forth in shown in SEQ ID NOs: 4, 8, and 12, and conservative sequence modifications thereof.

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99. A pharmaceutical composition comprising the expression vector of any one of claims 95-98 and a pharmaceutically acceptable carrier.

100. An anti-idiotypic antibody binding to an antibody of any one of claims 1-45.

101. An anti-idiotypic antibody binding to 2F2, 11B8 or 7D8.

102. Use of an anti-idiotypic antibody of claim 100 or 101 for detecting the level of human monoclonal antibody against CD20 in a sample.

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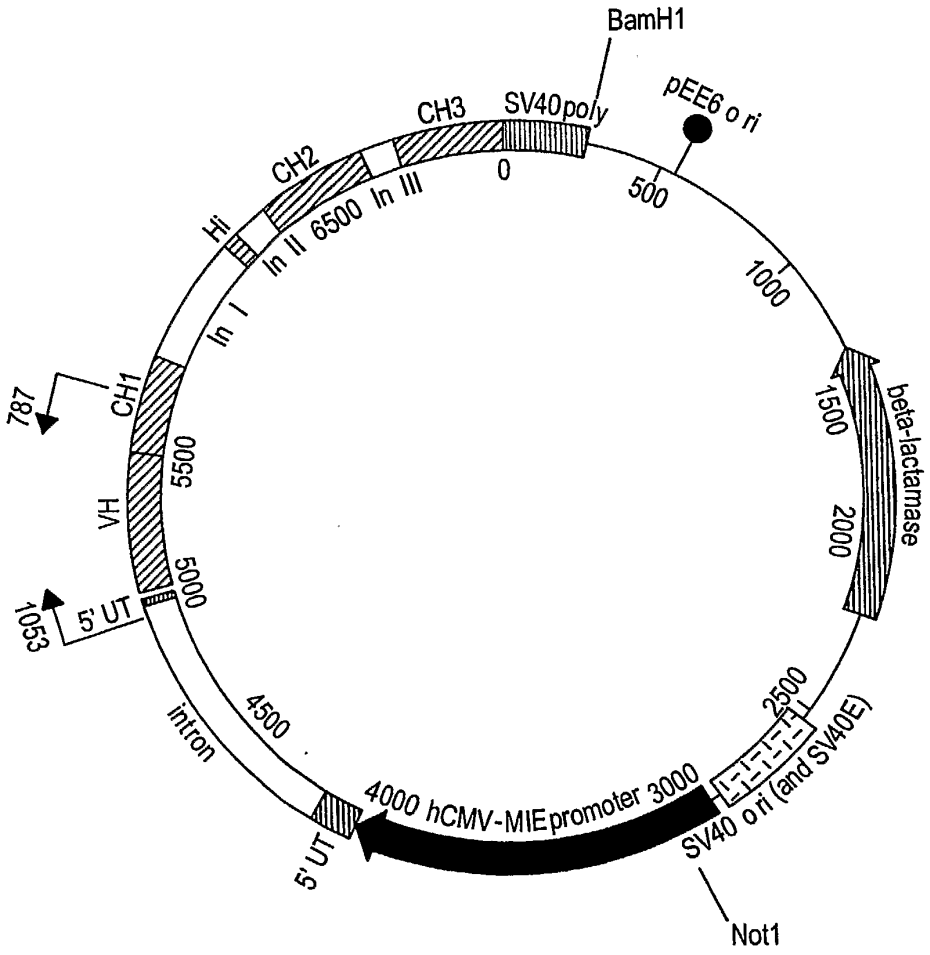


Fig. 1

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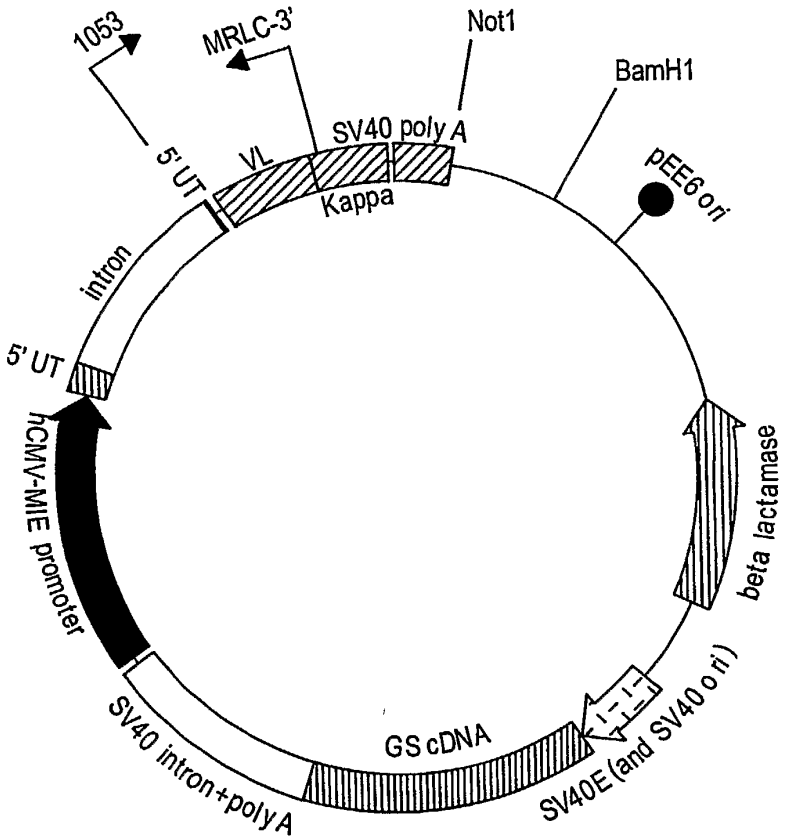


Fig. 2

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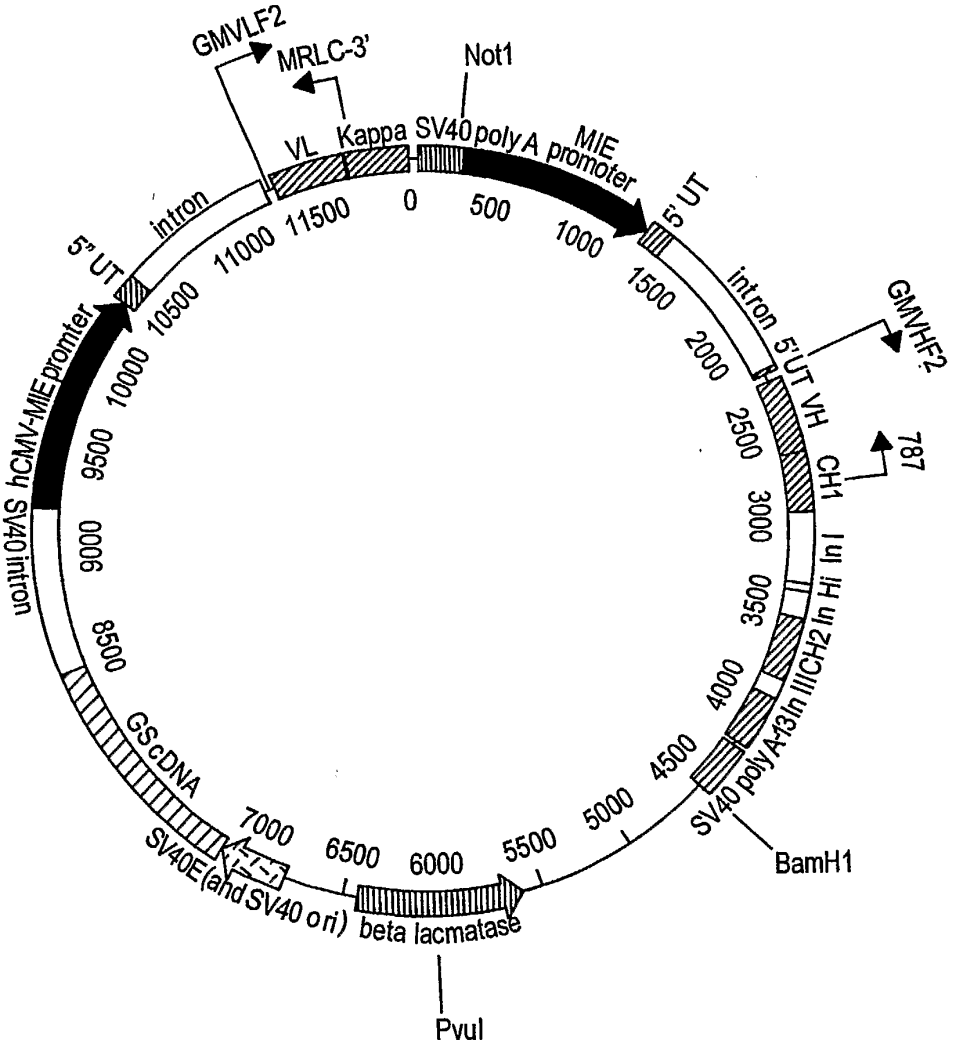


Fig. 3

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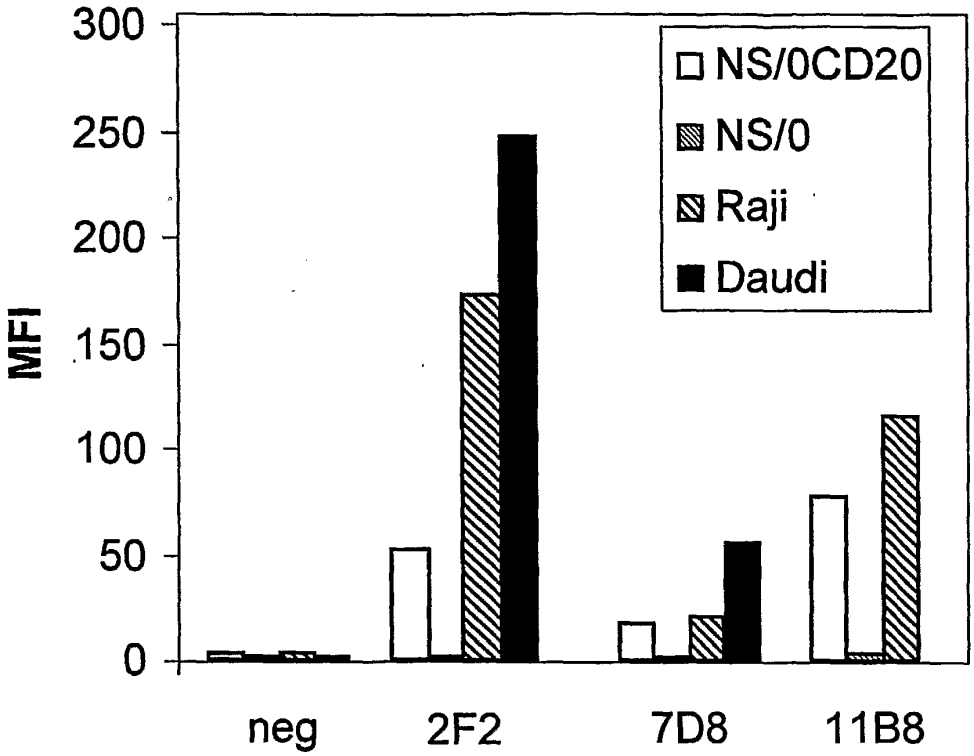


Fig. 4

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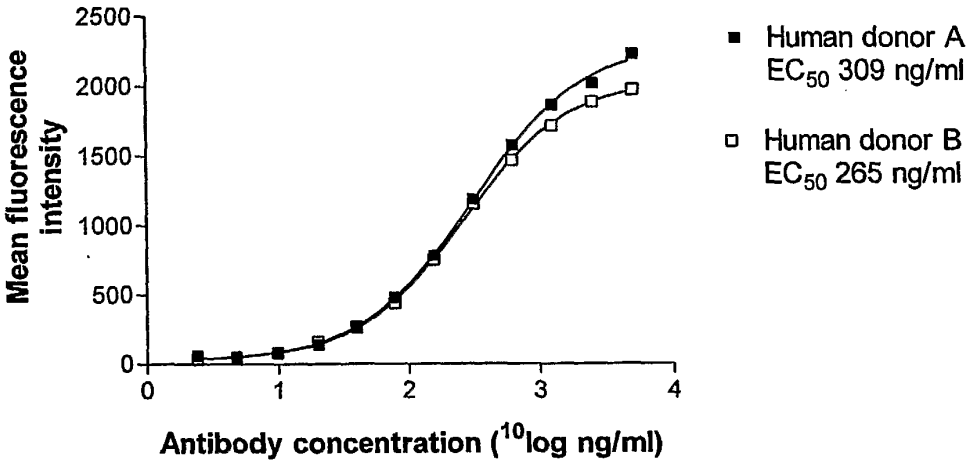

Mean fluorescence intensity
2500
2000
1500
1000
500
0
0
1
2
3
4
Antibody concentration (^{10}log ng/ml)
Human donor A
EC$_{50}$ 309 ng/ml
Human donor B
EC$_{50}$ 265 ng/ml

Fig. 5A

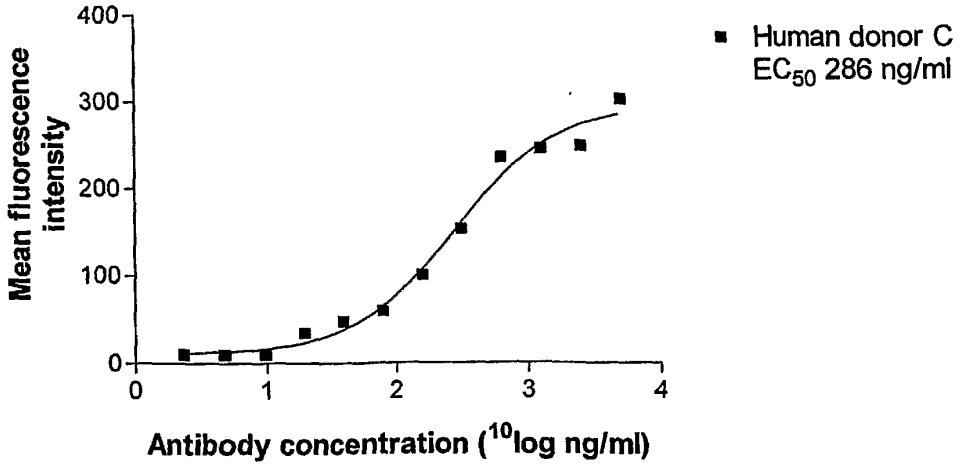

Mean fluorescence intensity
400
300
200
100
0
0
1
2
3
4
Antibody concentration (^{10}log ng/ml)
Human donor C
EC$_{50}$ 286 ng/ml

Fig. 5B

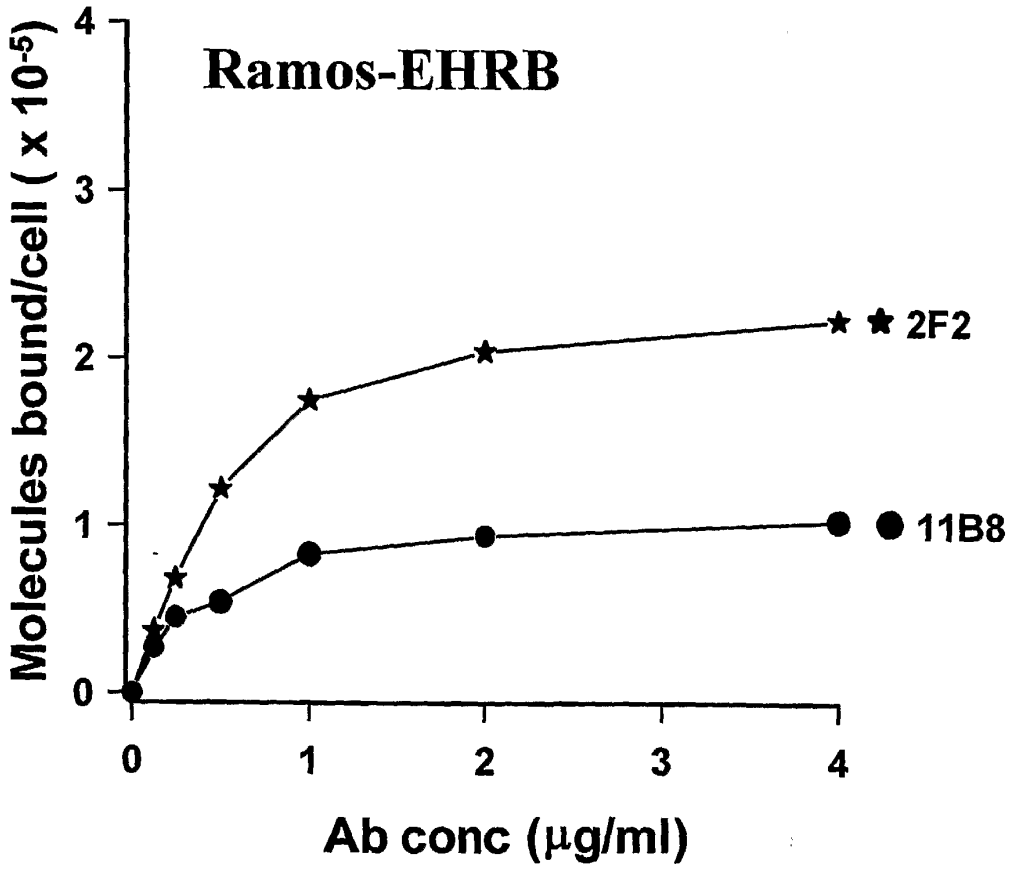

Ramos-EHRB
Molecules bound/cell (x 10-5)
4
3
2
1
0
0
1
2
3
4
Ab conc (μg/ml)
2F2
11B8

Fig 6

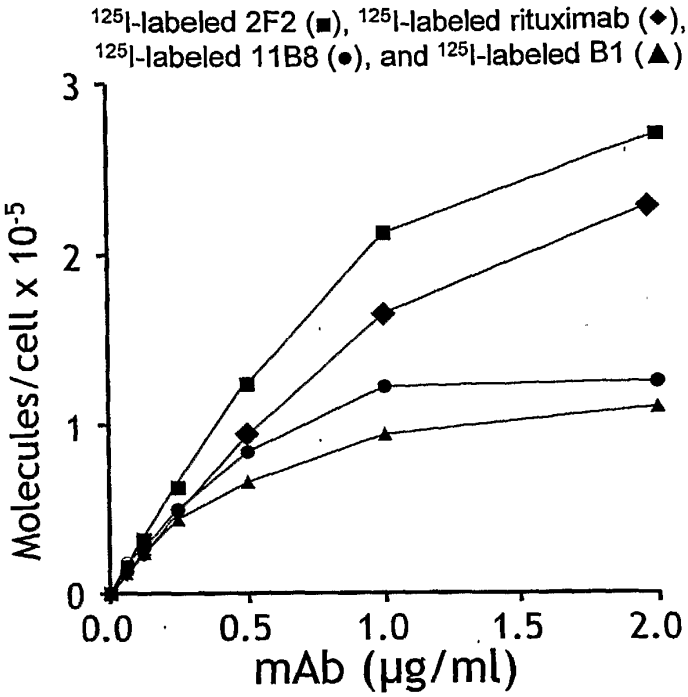

^{125}I-labeled 2F2 (■), ^{125}I-labeled rituximab (◆),
^{125}I-labeled 11B8 (●), and ^{125}I-labeled B1 (▲)
Molecules/cell x 10^{-5}
0
1
2
3
0.0
0.5
1.0
1.5
2.0
mAb (µg/ml)

Fig. 7A

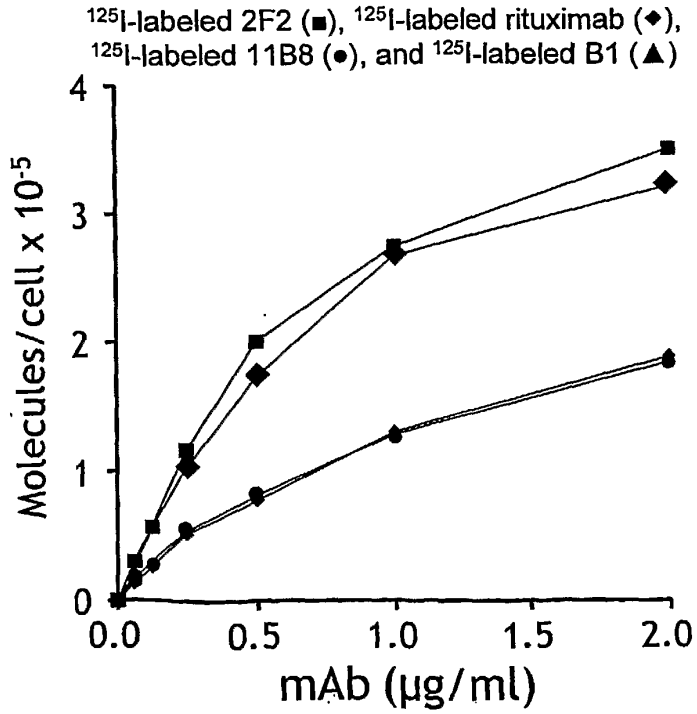

^{125}I-labeled 2F2 (■), ^{125}I-labeled rituximab (◆),
^{125}I-labeled 11B8 (●), and ^{125}I-labeled B1 (▲)
Molecules/cell x 10^{-5}
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
mAb (µg/ml)

Fig. 7B

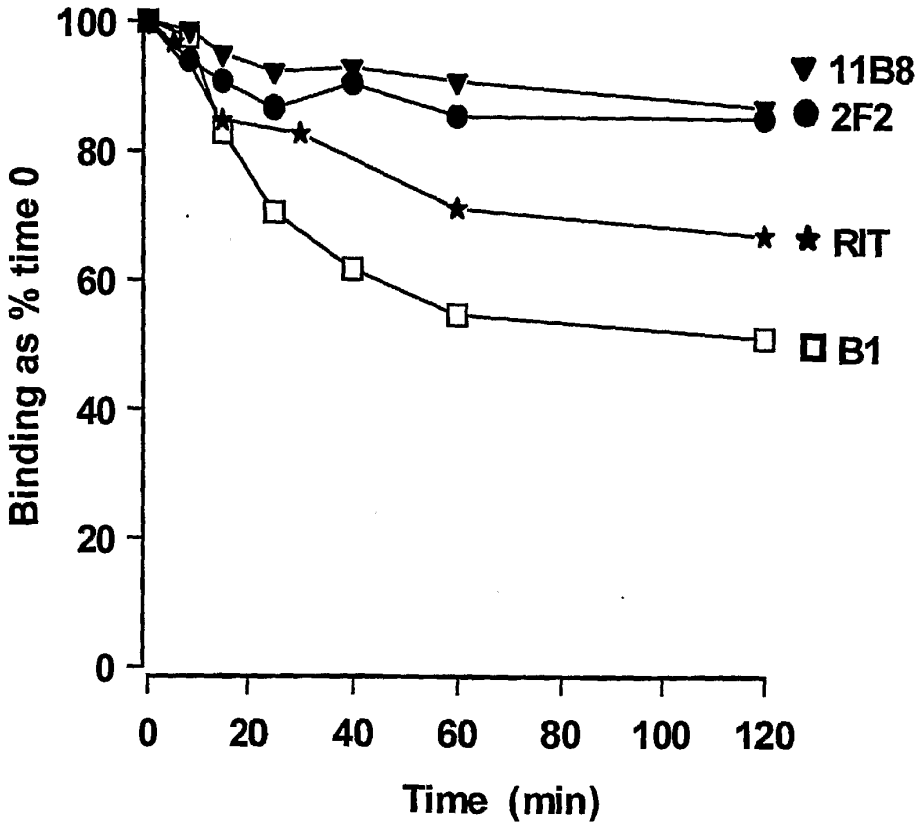


Fig. 8

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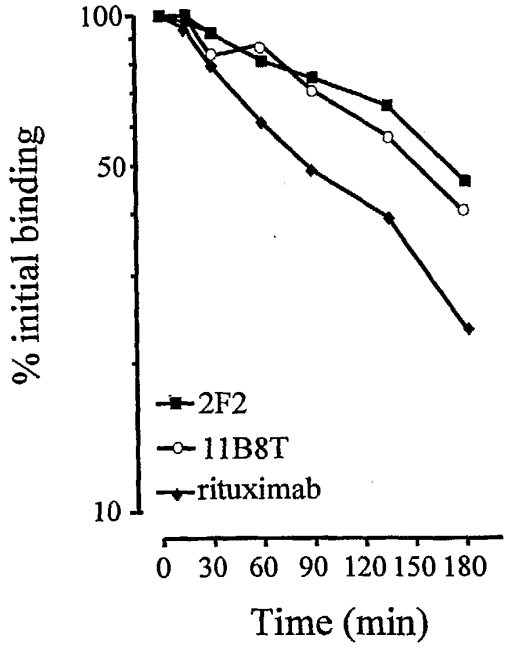


Fig. 9

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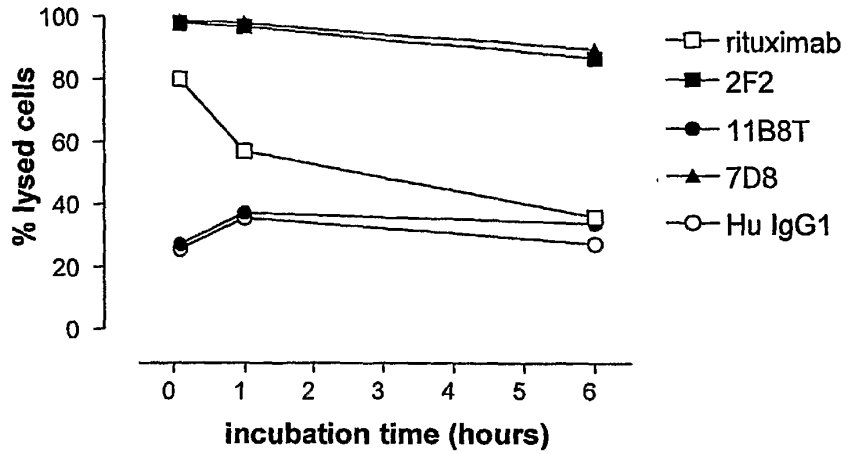


Fig. 10A

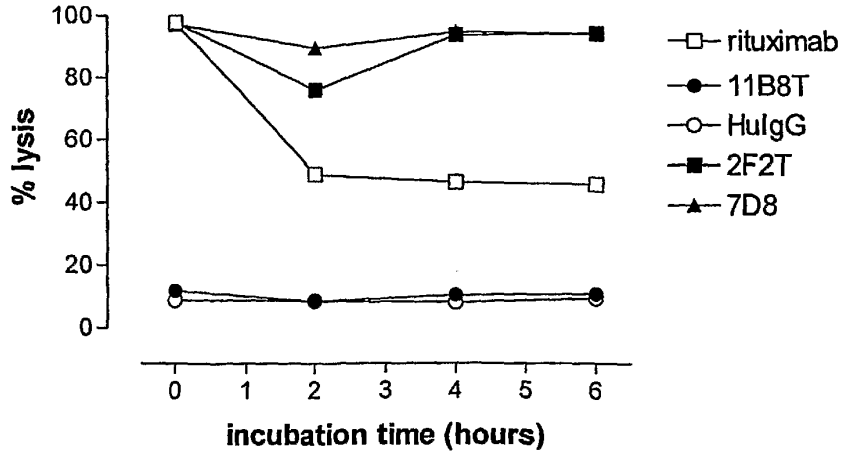


Fig. 10B

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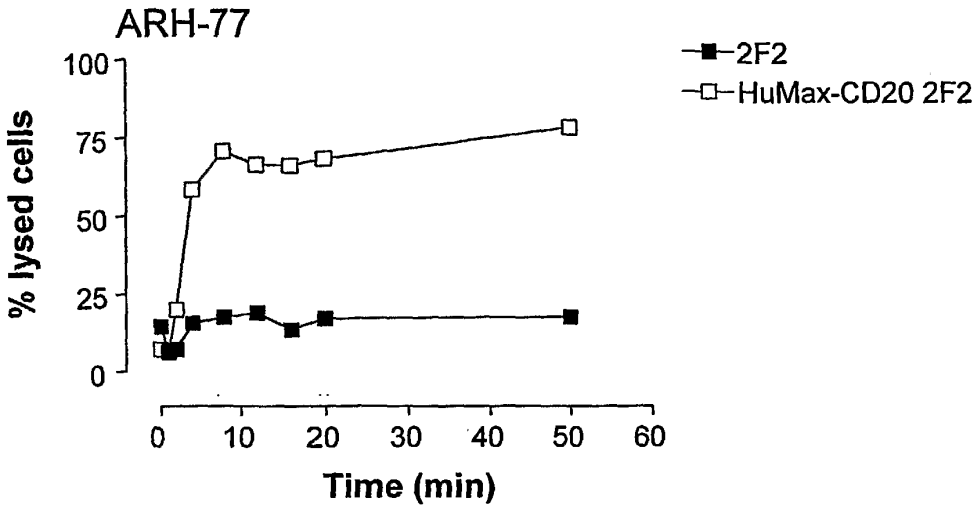


Fig. 11A

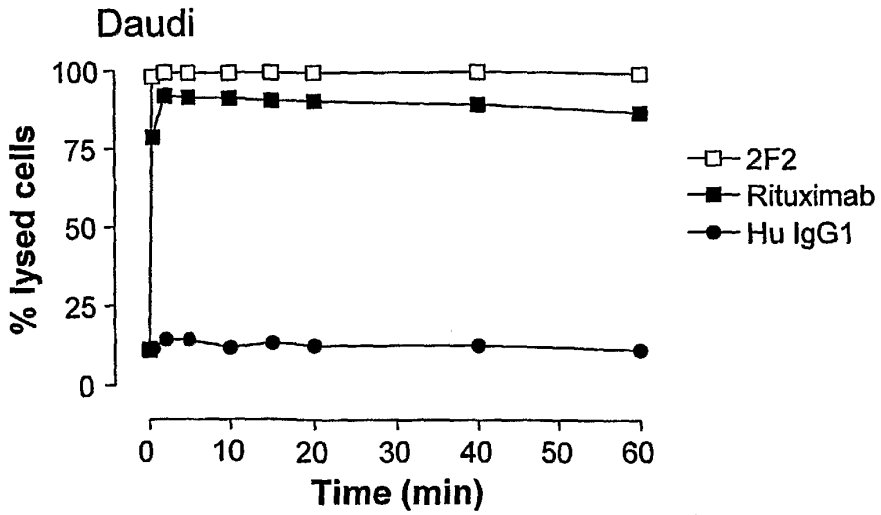


Fig. 11A

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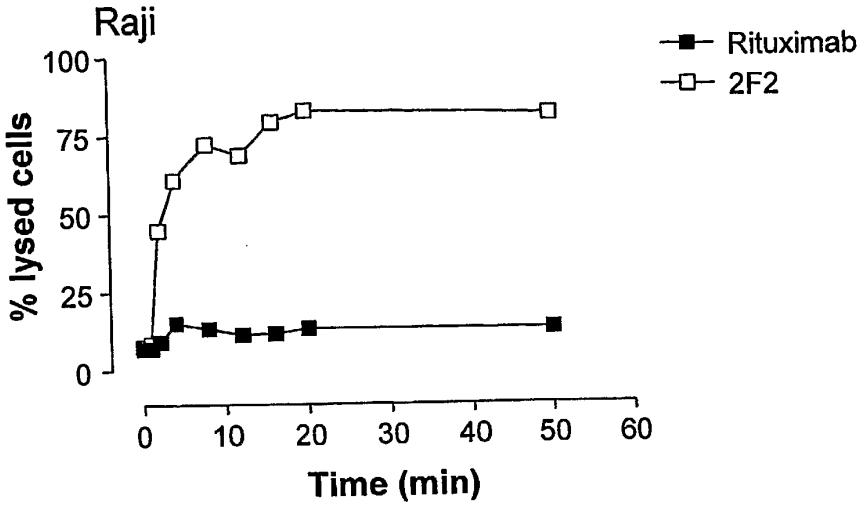


Fig. 11C

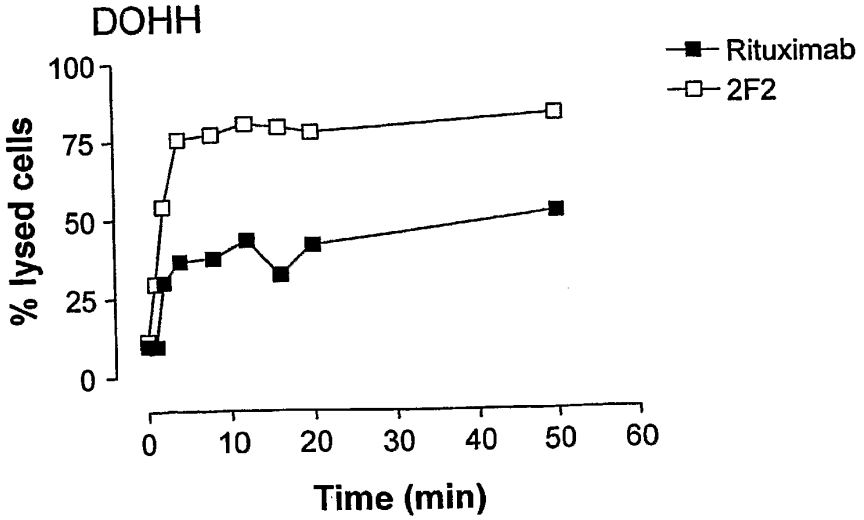


Fig. 11D

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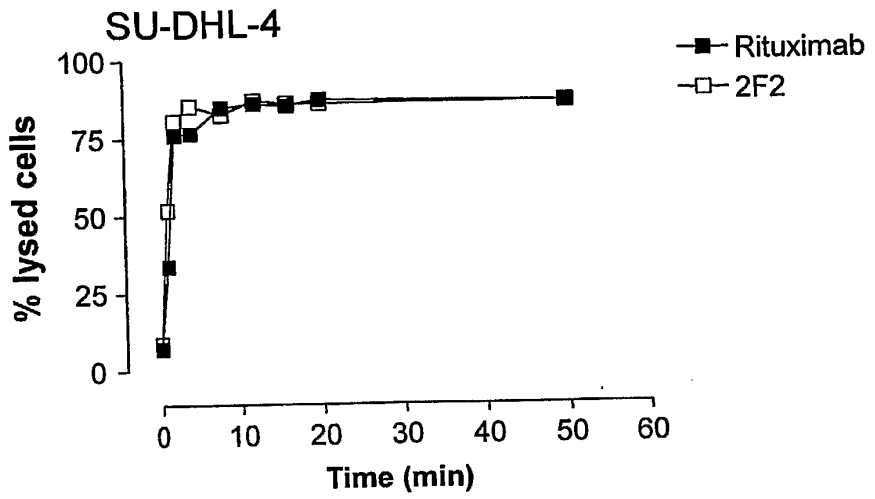


Fig. 11E

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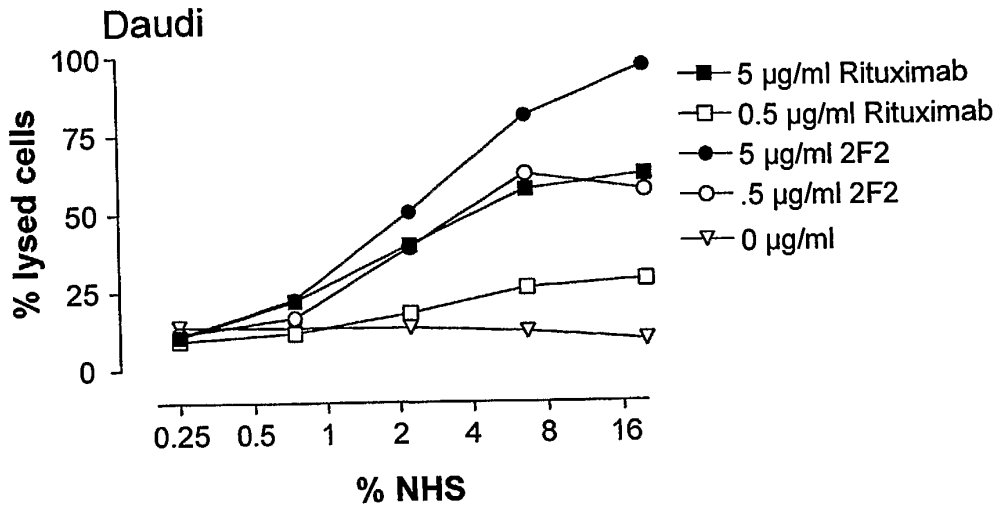


Fig. 12A

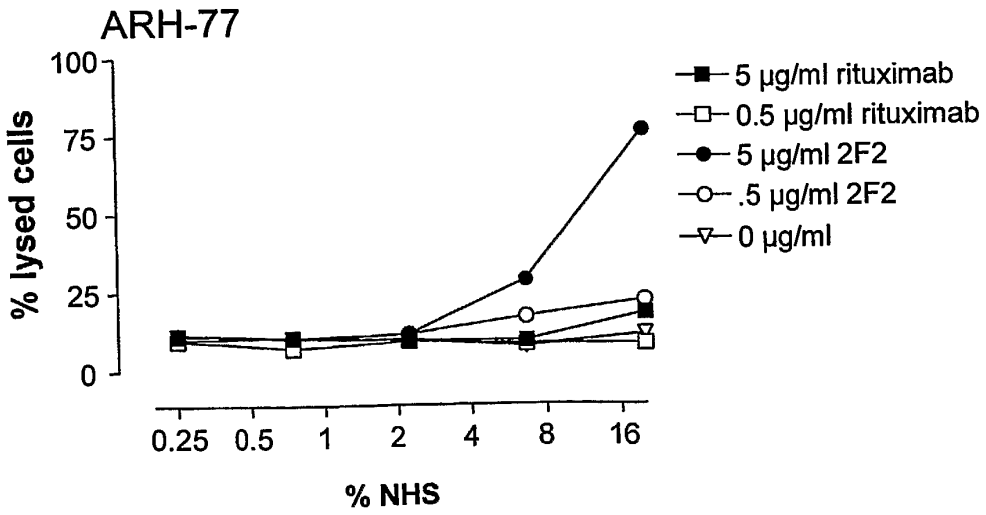


Fig. 12B

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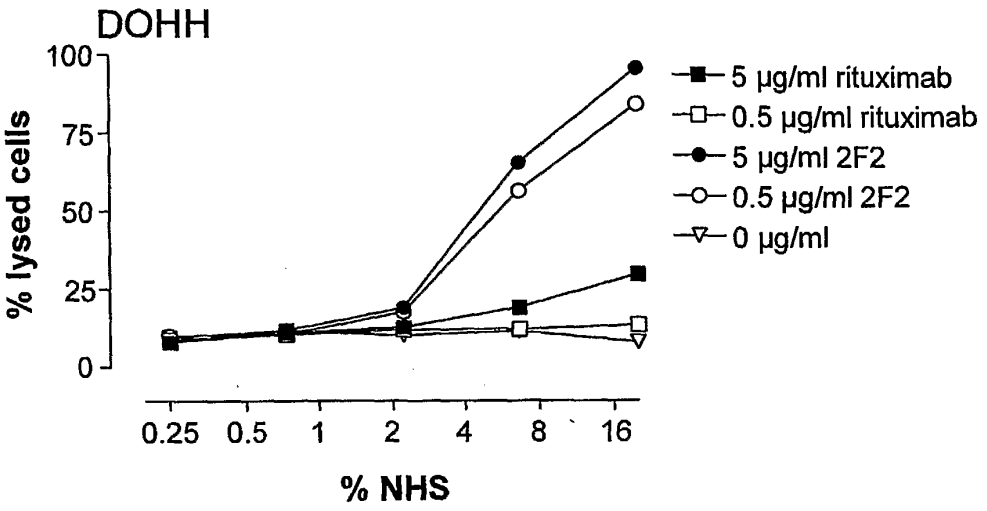


Fig. 12C

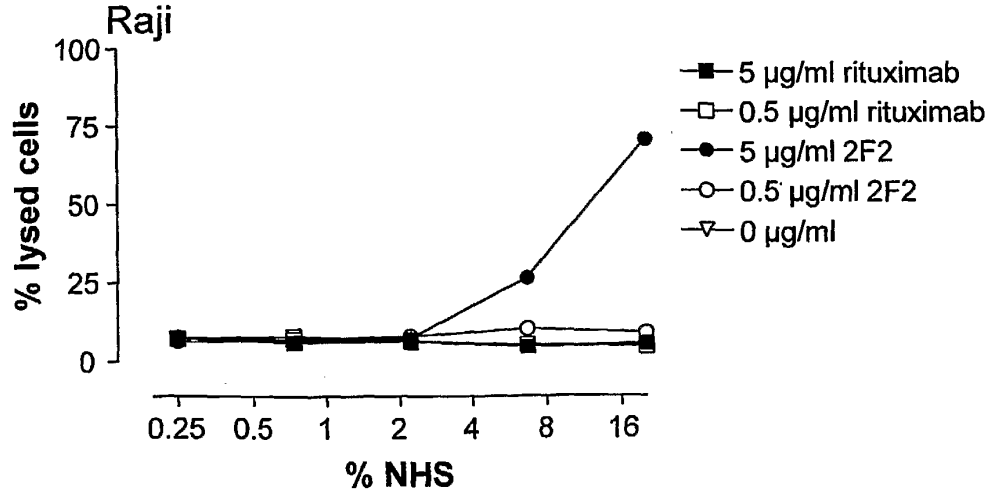


Fig. 12D

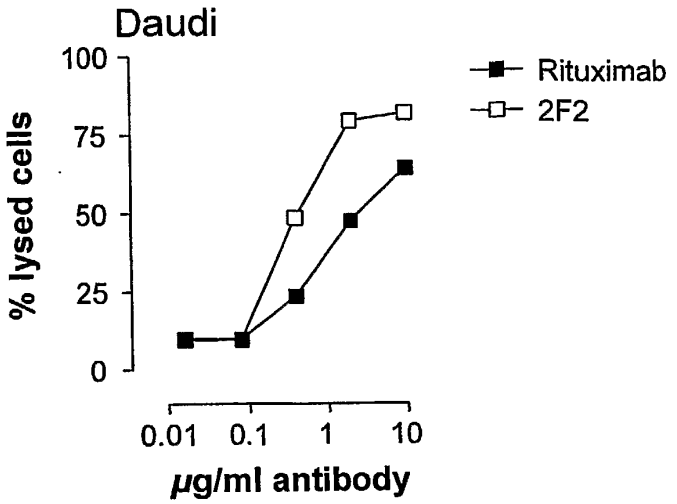


Fig. 13A

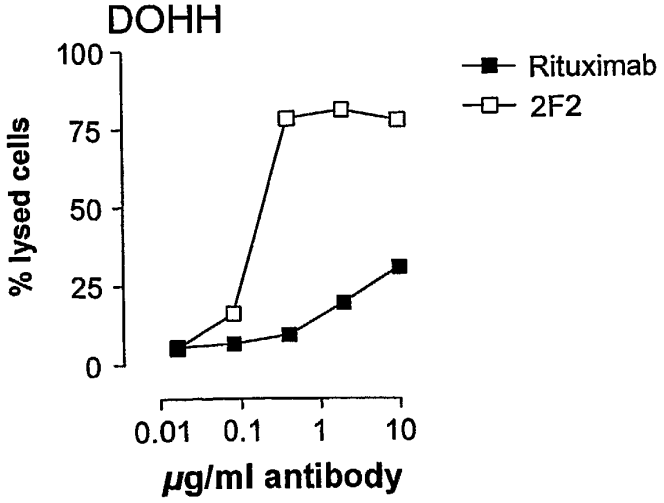


Fig. 13B

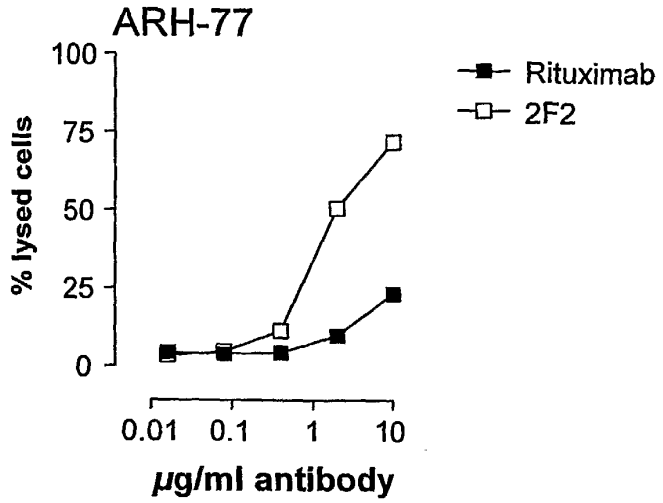


Fig. 13C

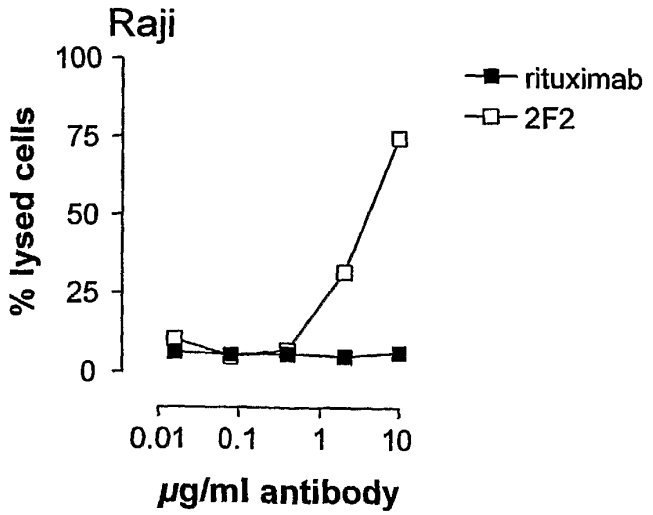


Fig. 13D

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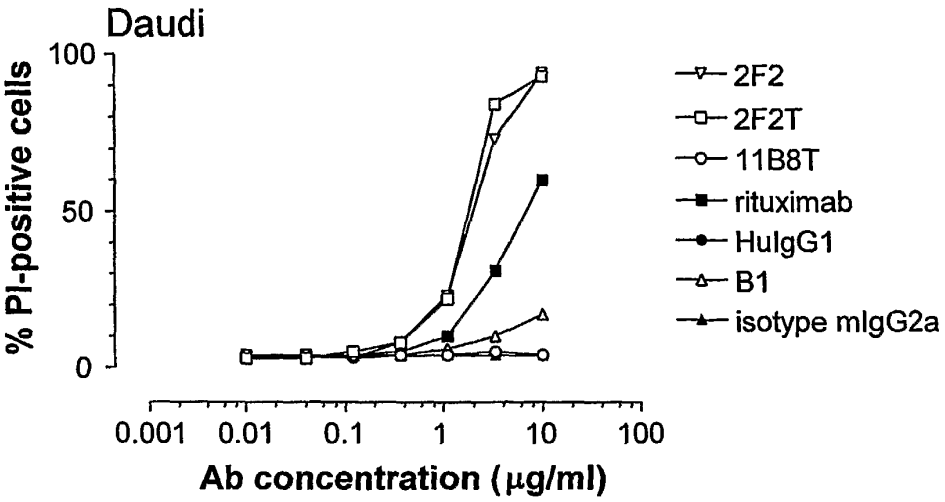


Fig. 14A

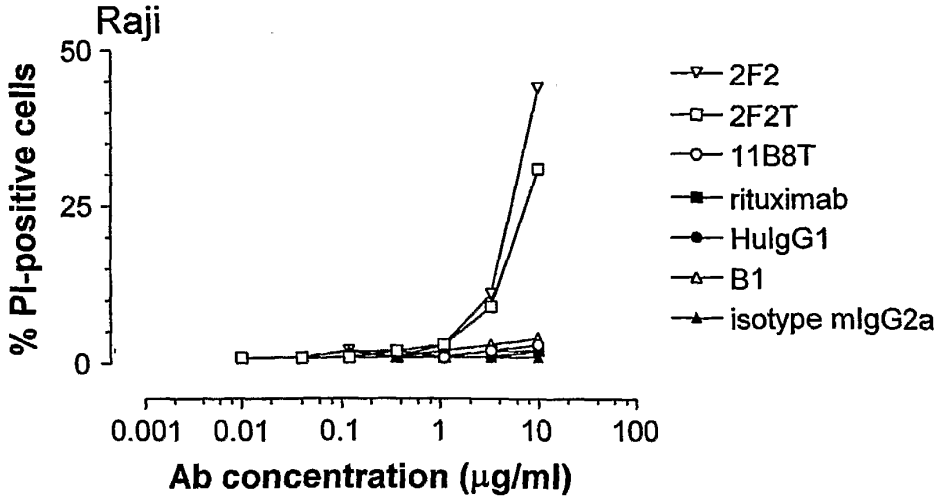


Fig. 14B

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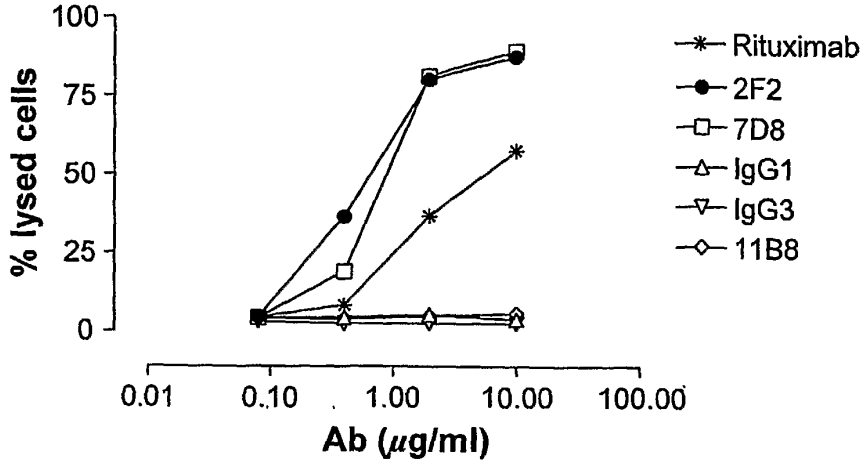


Fig. 15A

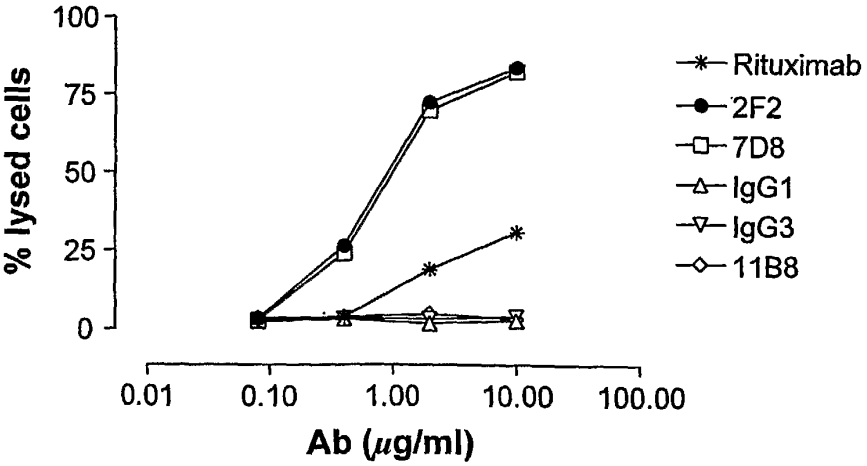


Fig. 15B

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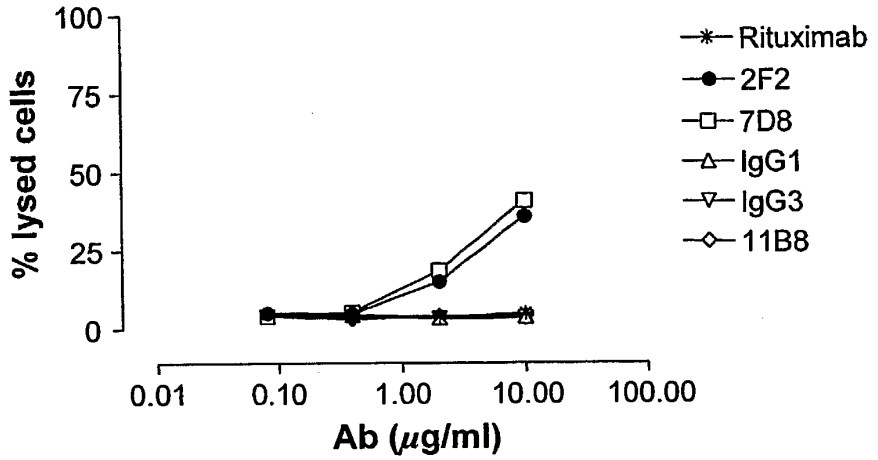


Fig. 16A

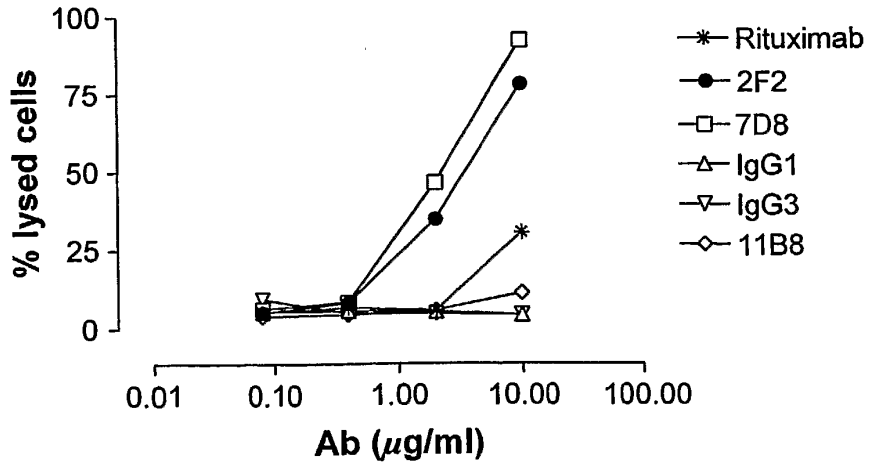


Fig. 16B

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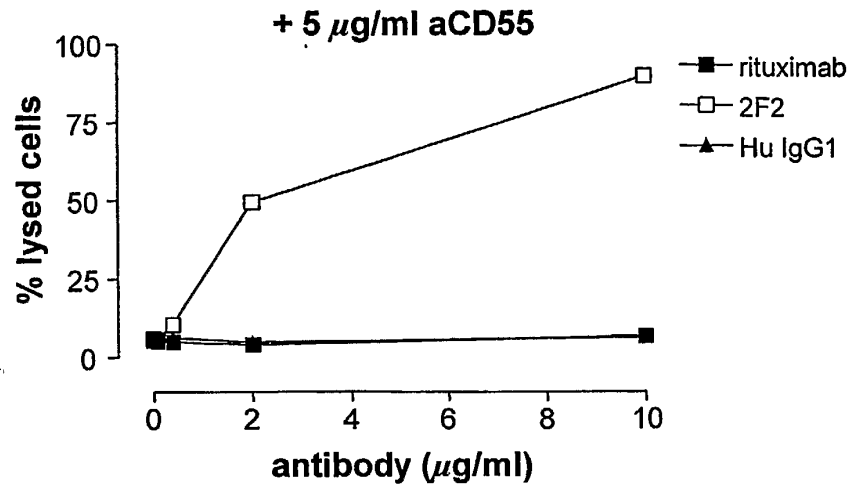


Fig. 17A

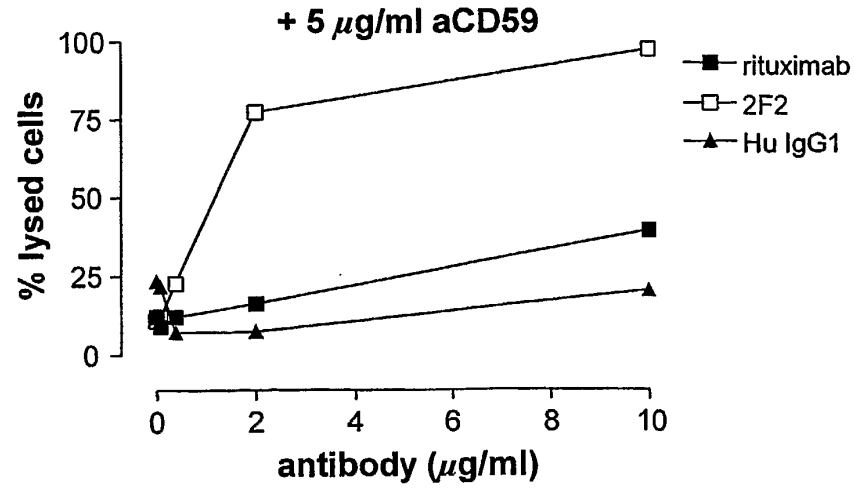


Fig. 17B

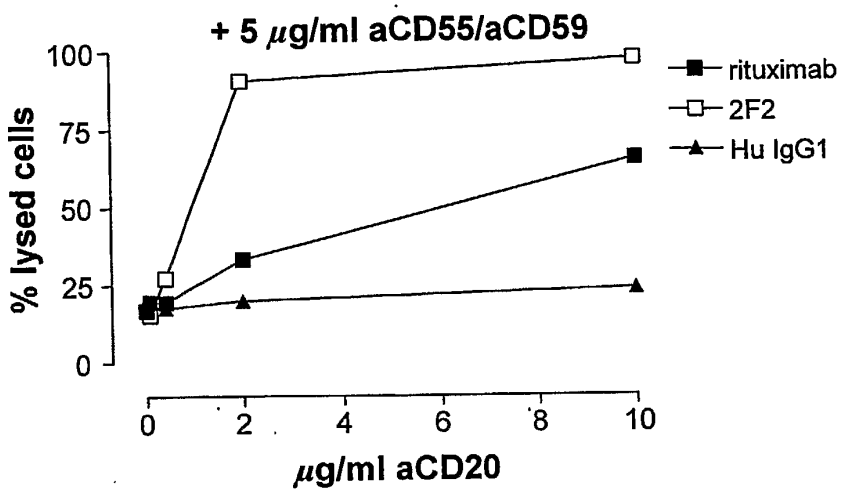


Fig. 17C

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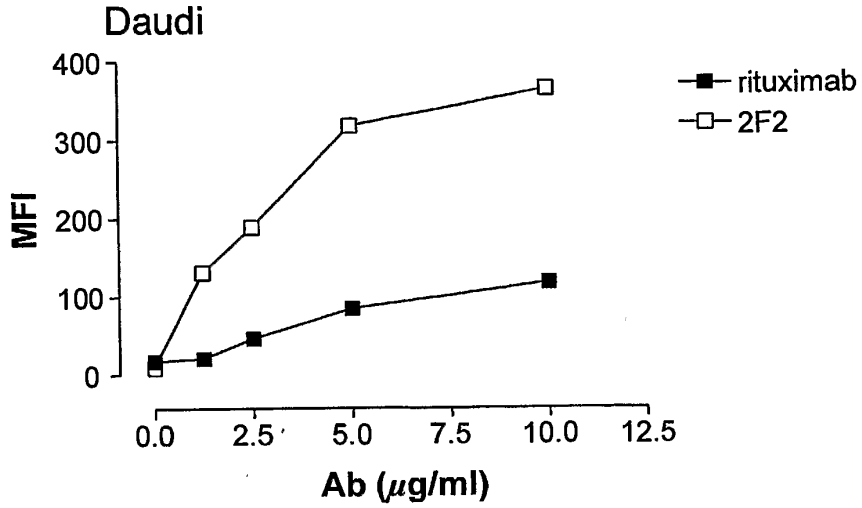


Fig. 18A

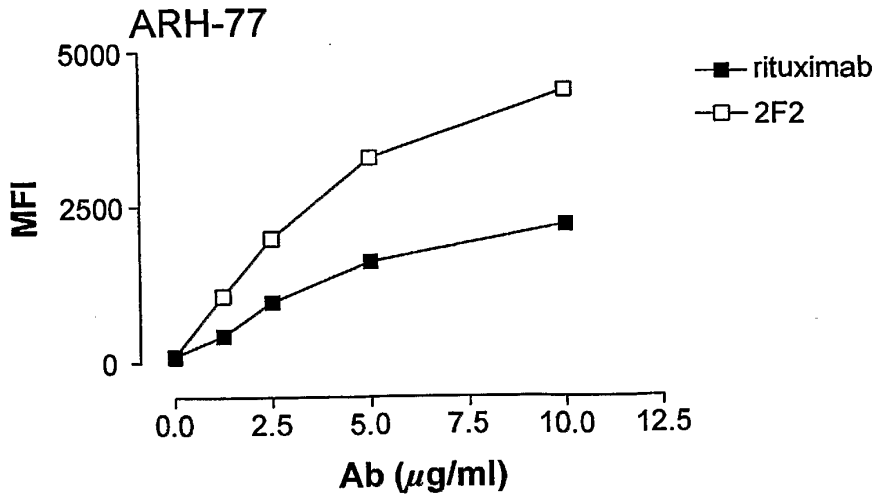


Fig. 18B

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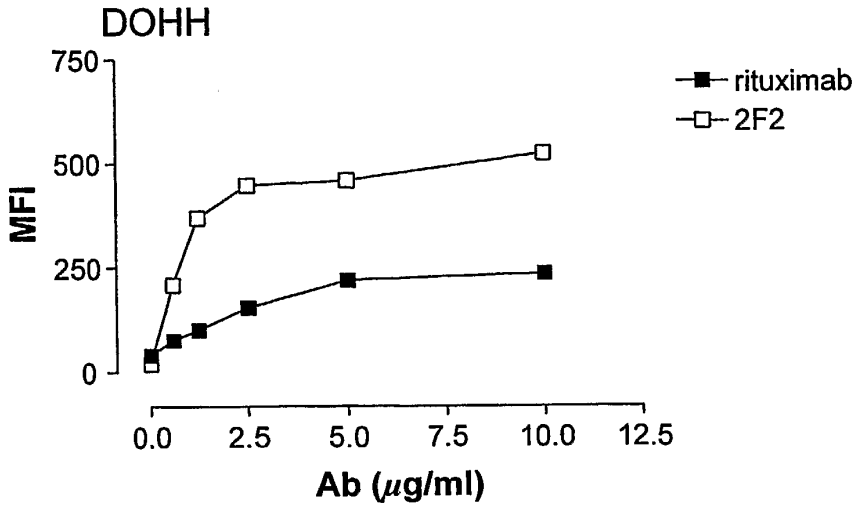


Fig. 18C

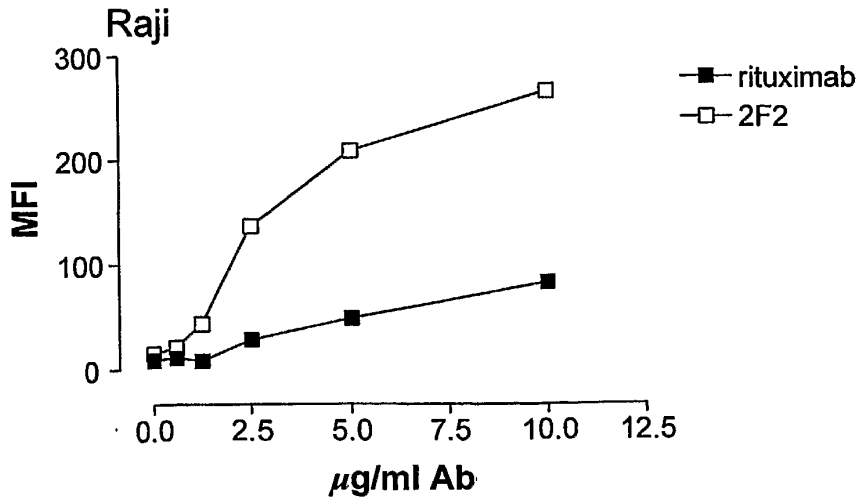


Fig. 18D

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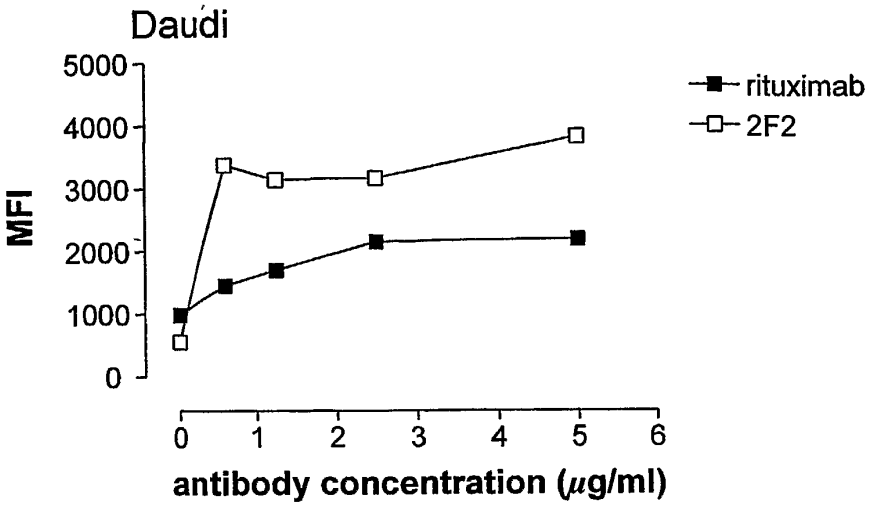


Fig. 19A

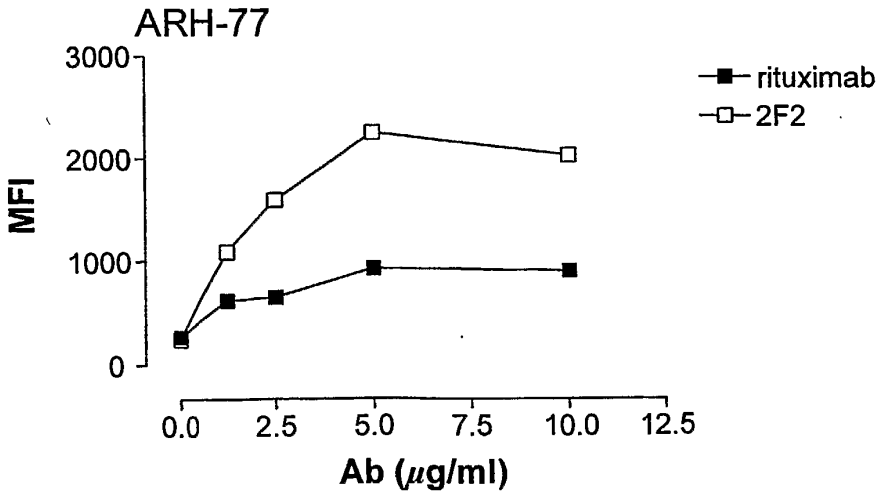


Fig. 19B

Samsung Bioepis Exhibit No. 1028
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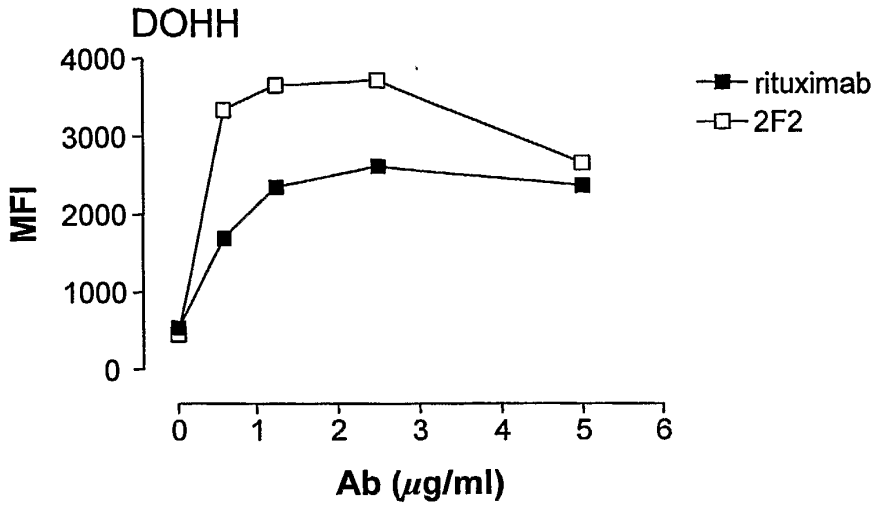


Fig. 19C

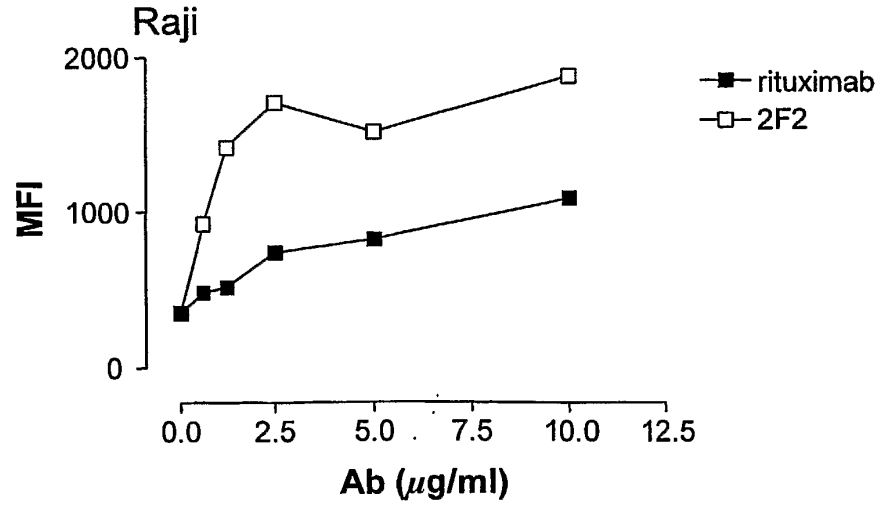


Fig. 19D

Samsung Bioepis Exhibit No. 1028
Page 740

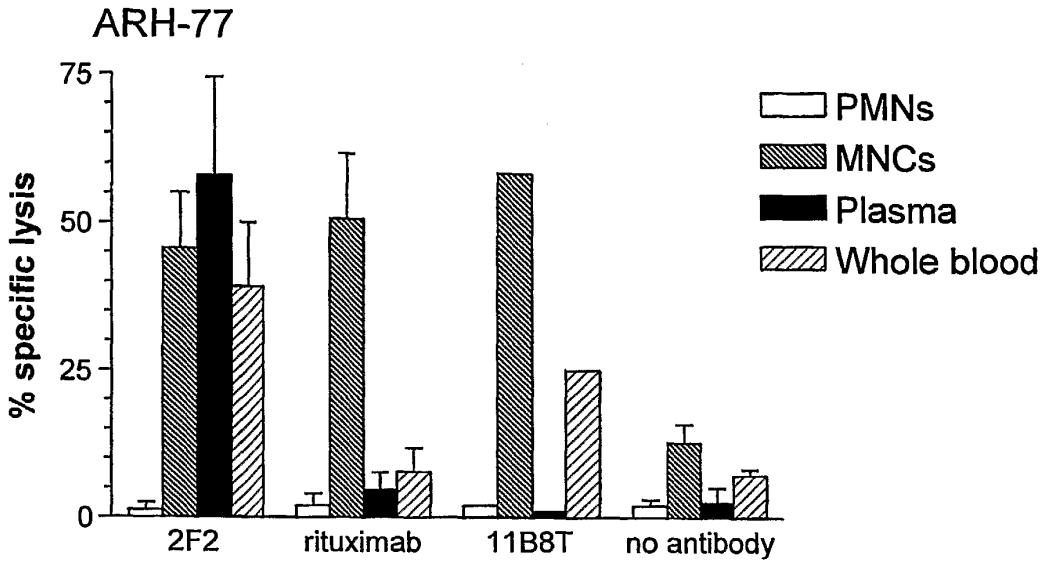


Fig. 20

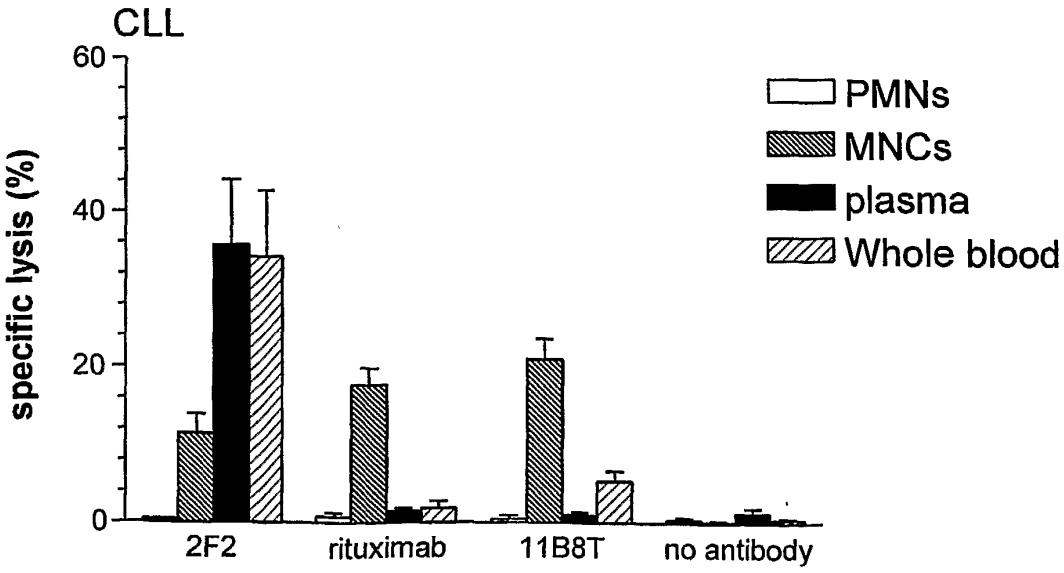


Fig. 21

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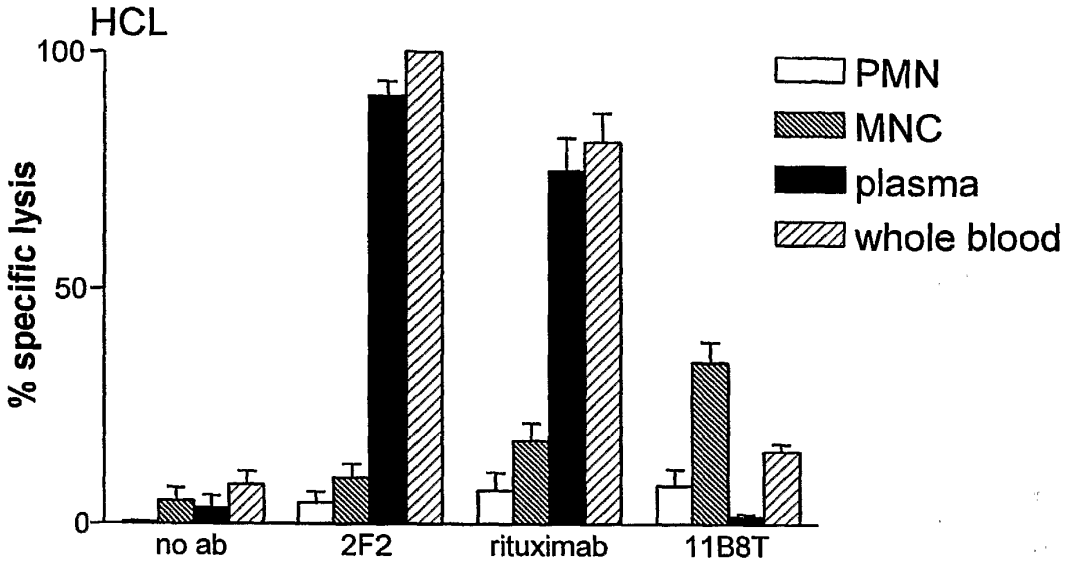

HCL
100
50
0
% specific lysis
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab
11B8T

Fig. 22

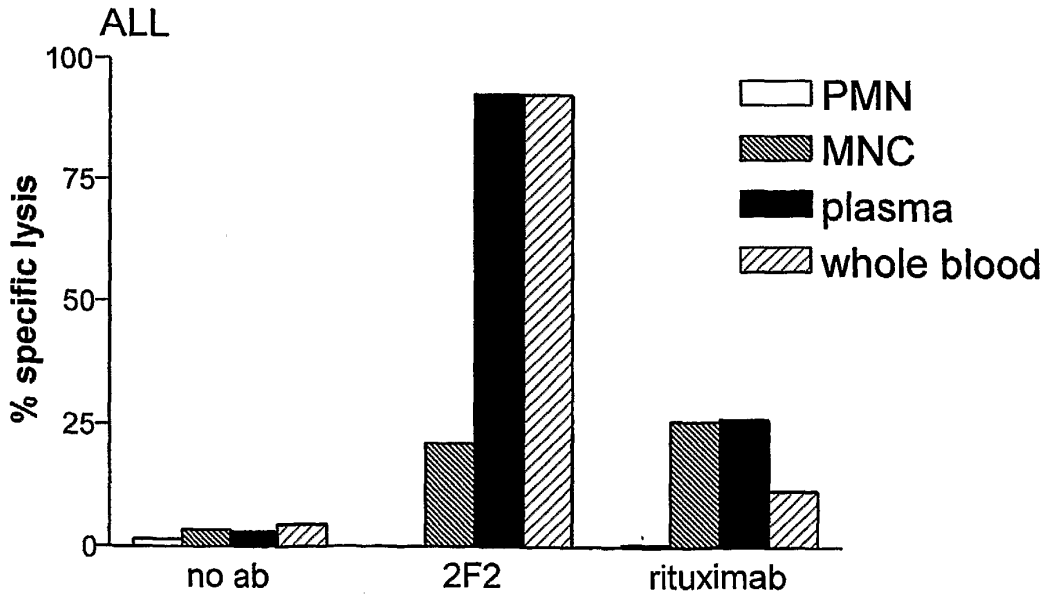

ALL
100
75
50
25
0
% specific lysis
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab

Fig. 23

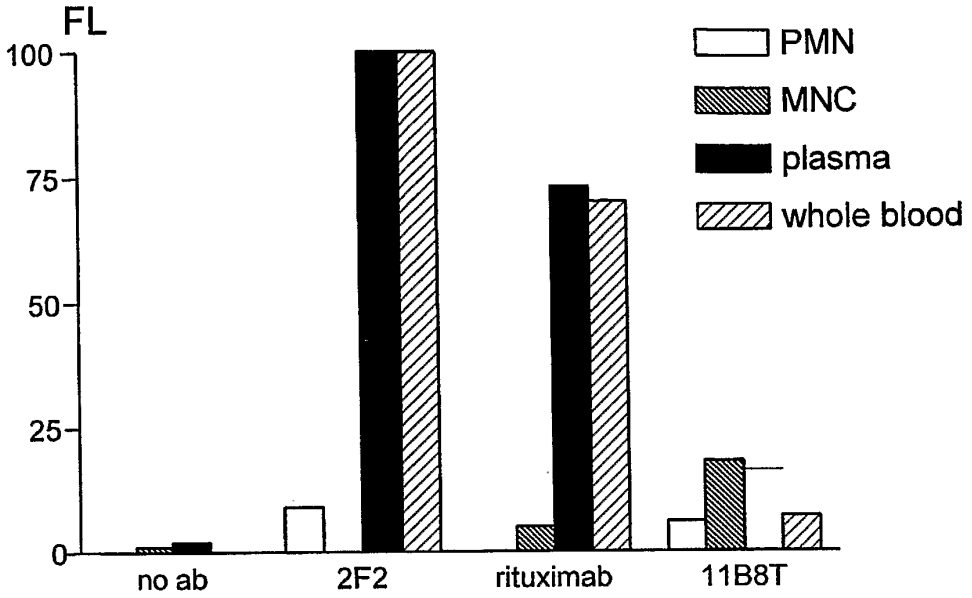

FL
100
75
50
25
0
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab
11B8T

Fig. 24

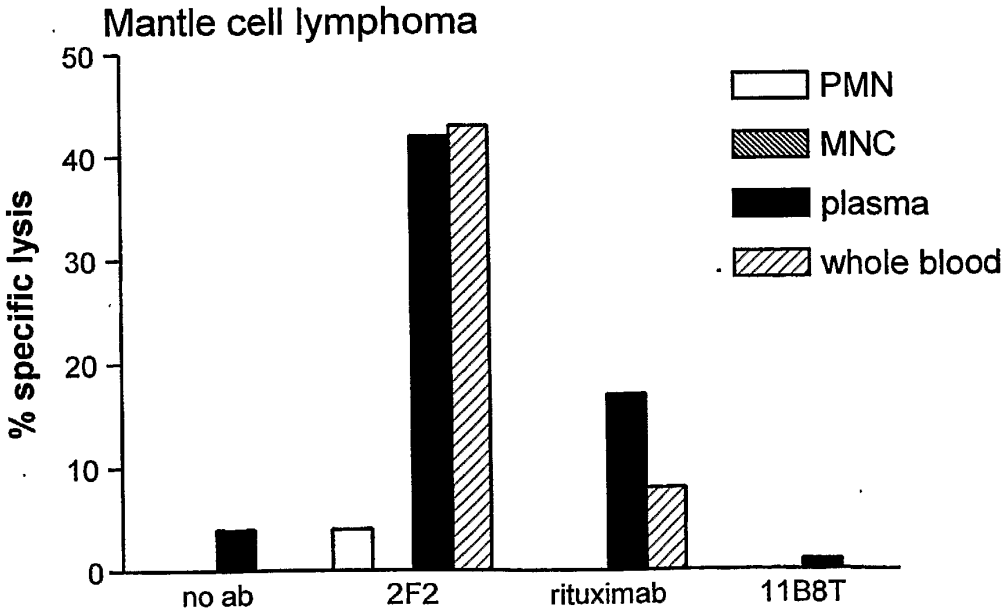

Mantle cell lymphoma
% specific lysis
50
40
30
20
10
0
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab
11B8T

Fig. 25

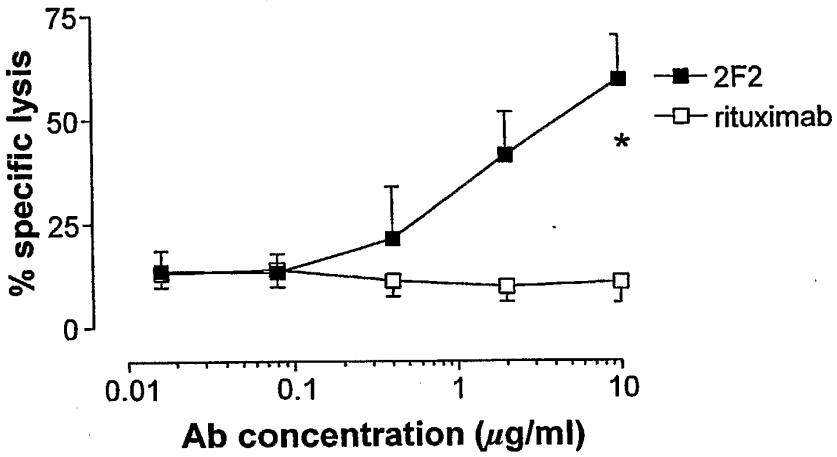


Fig. 26

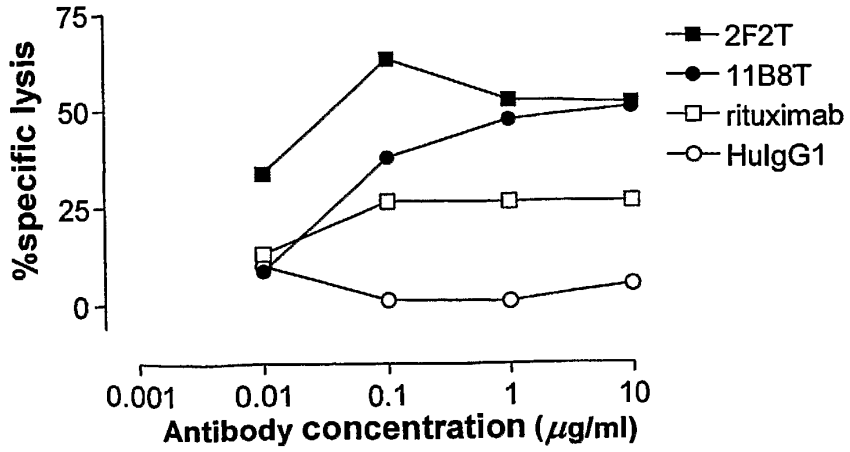


Fig. 27

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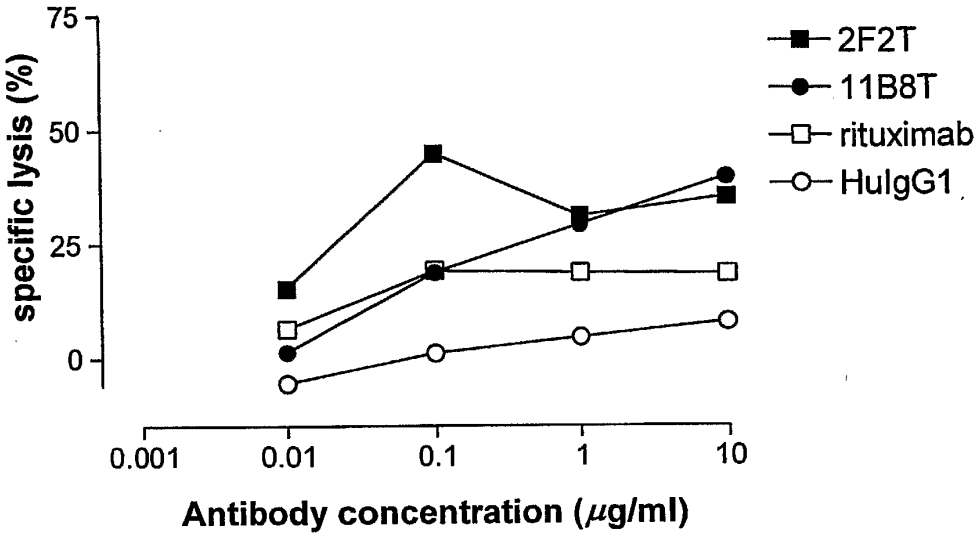


Fig. 28

Samsung Bioepis Exhibit No. 1028
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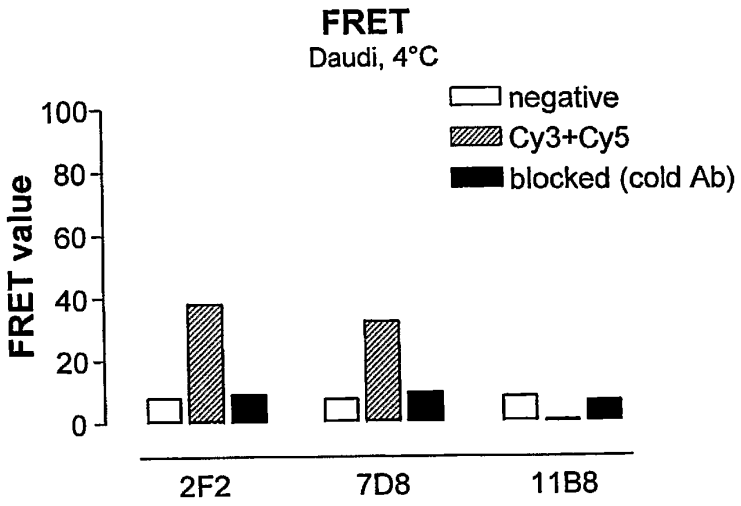


Fig. 29A

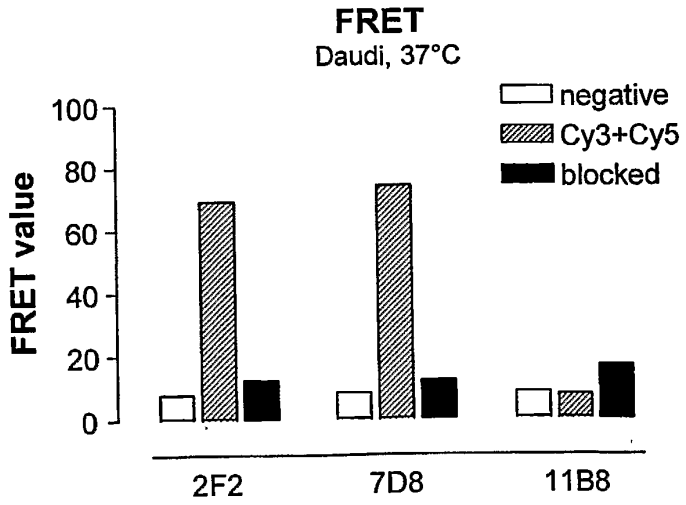


Fig. 29B

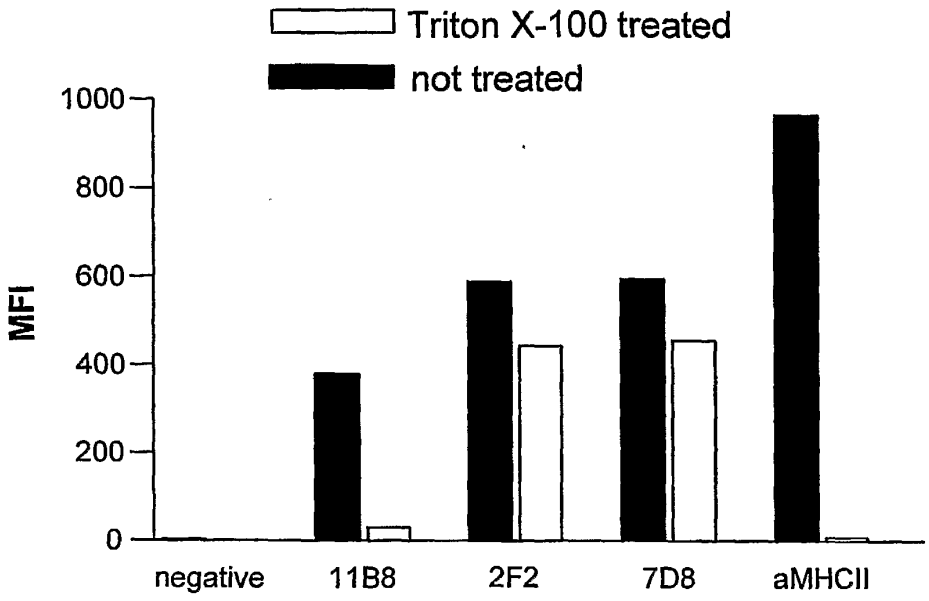


Fig. 29C

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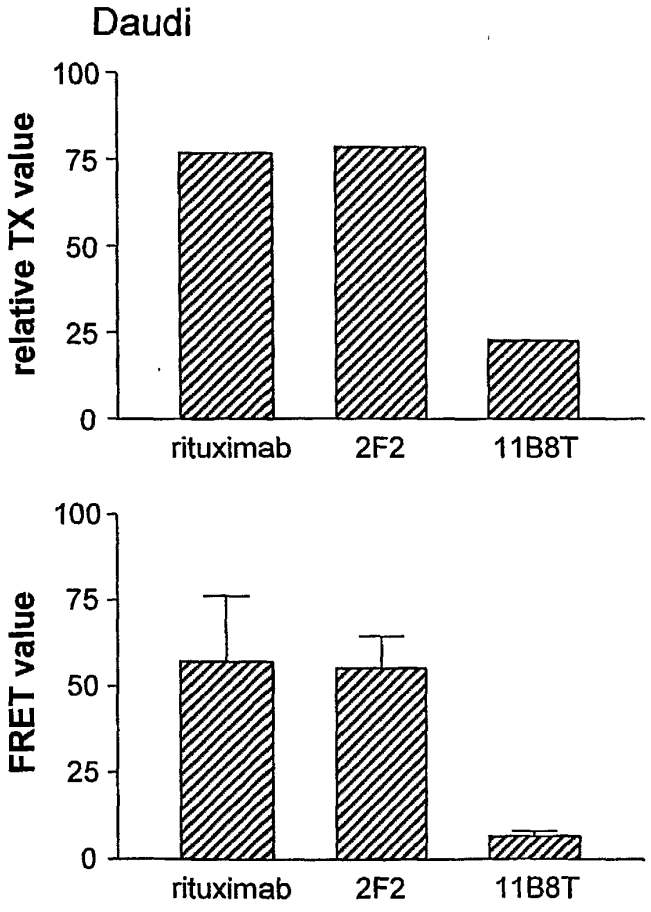

Daudi
relative TX value
100
75
50
25
0
rituximab
2F2
11B8T
FRET value
100
75
50
25
0
rituximab
2F2
11B8T

Fig. 30

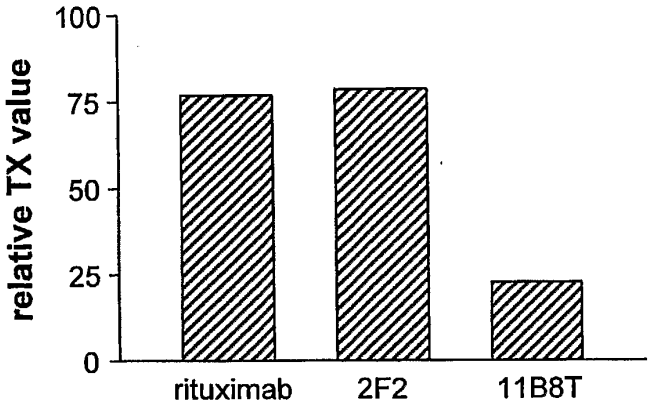


Fig. 31

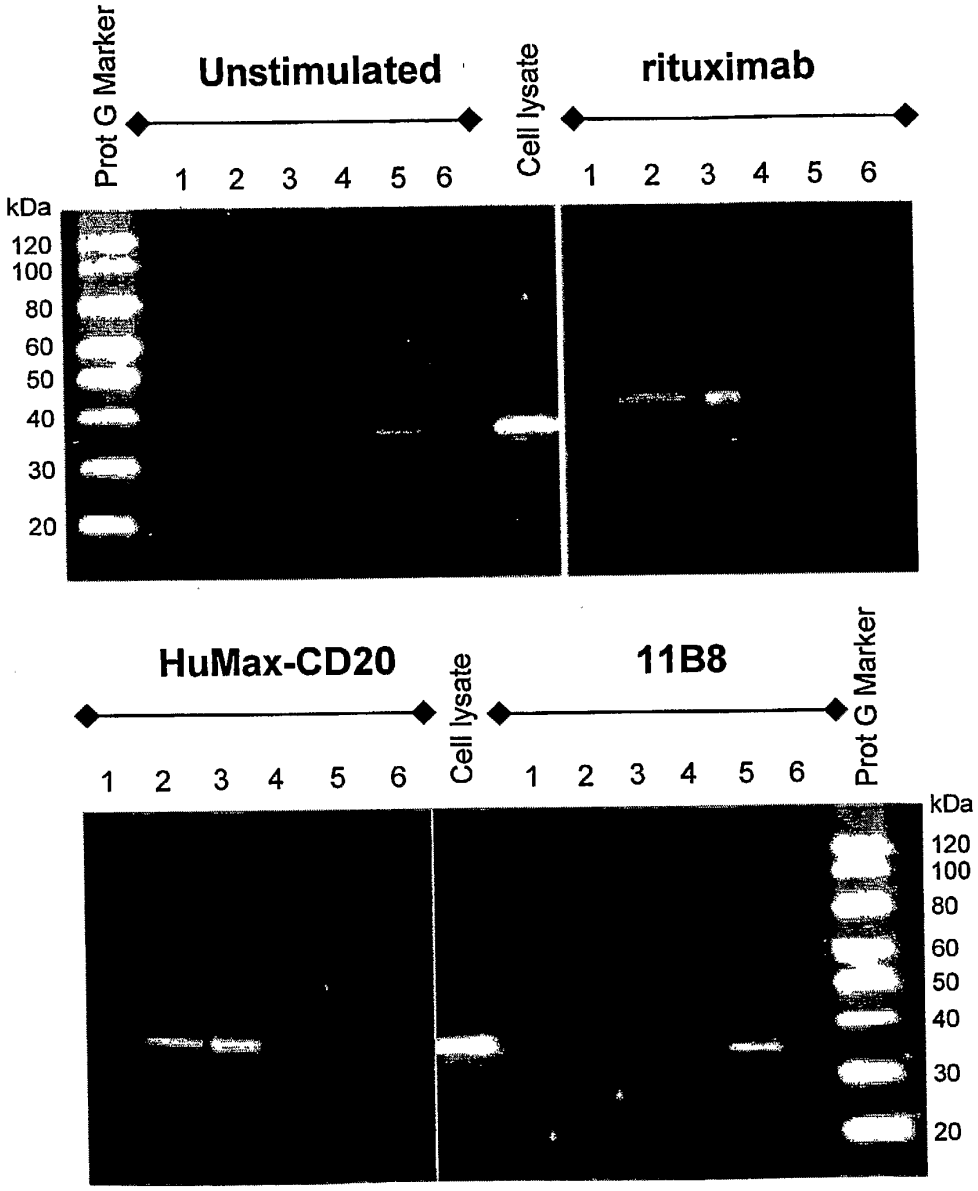

Prot G Marker
Unstimulated
Cell lysate
rituximab
1 2 3 4 5 6
1 2 3 4 5 6
kDa
120
100
80
60
50
40
30
20
HuMax-CD20
Cell lysate
11B8
Prot G Marker
1 2 3 4 5 6
1 2 3 4 5 6
kDa
120
100
80
60
50
40
30
20

Fig. 32

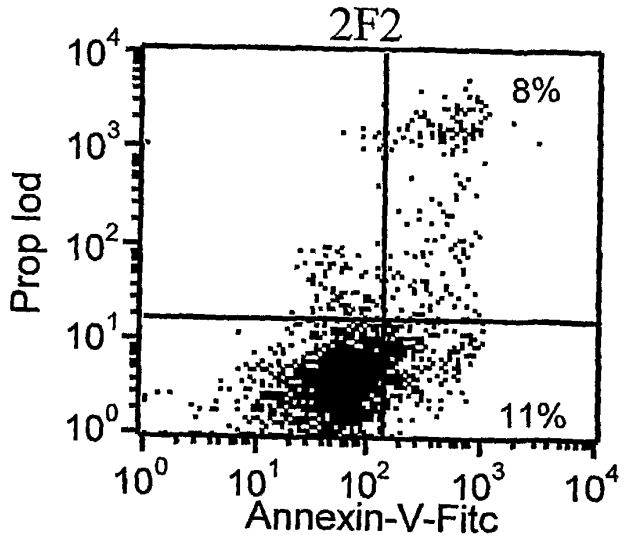


Fig. 33A

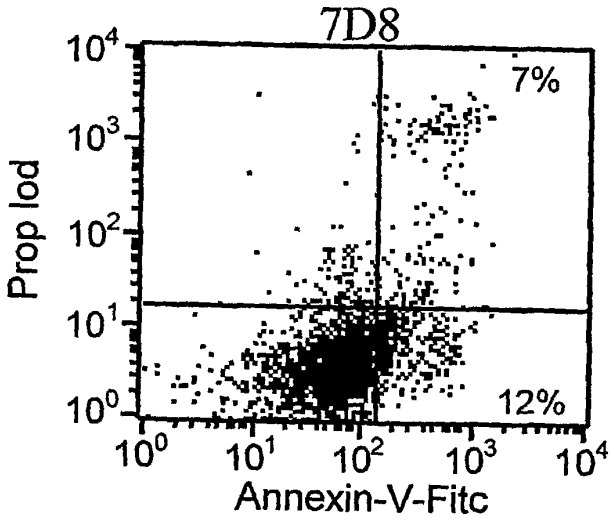


Fig. 33B

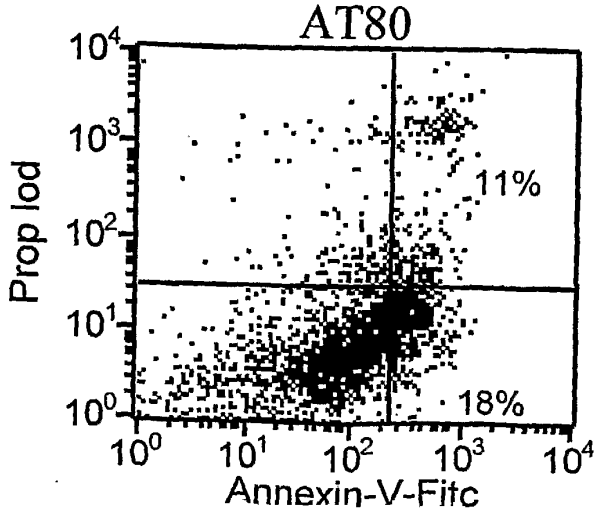


Fig. 33C

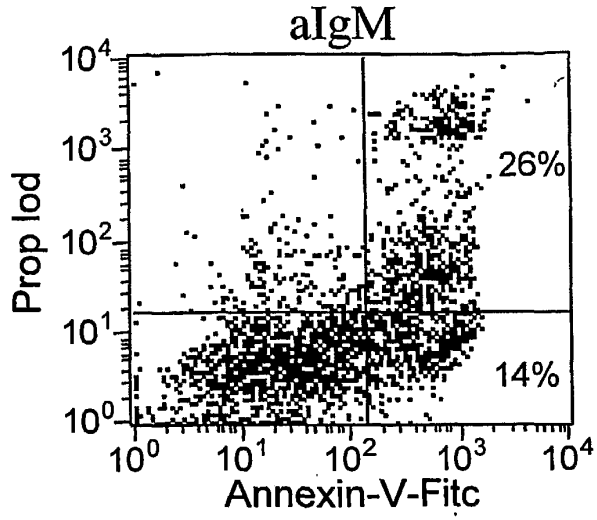


Fig. 33D

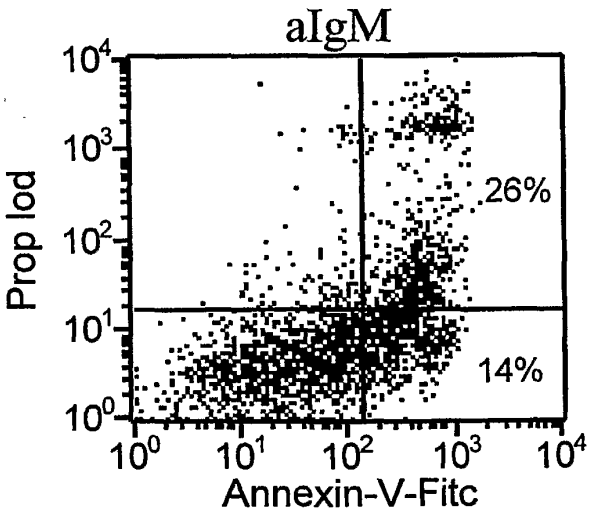


Fig. 33E

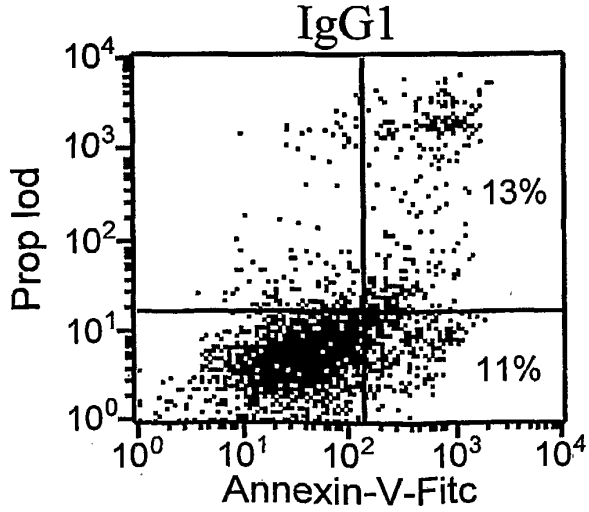


Fig. 33F

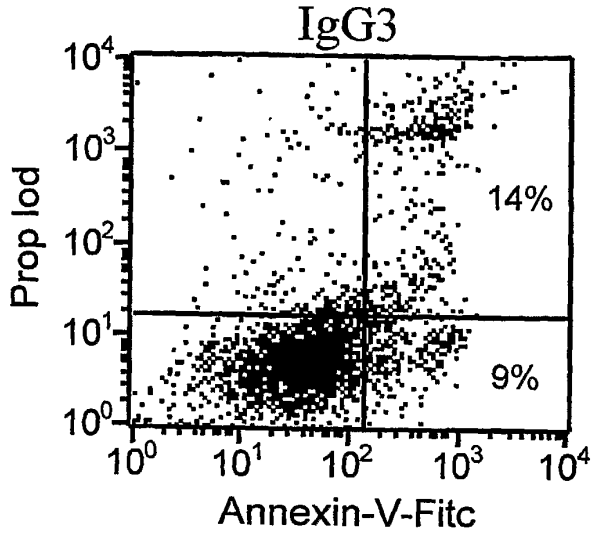


Fig. 33G

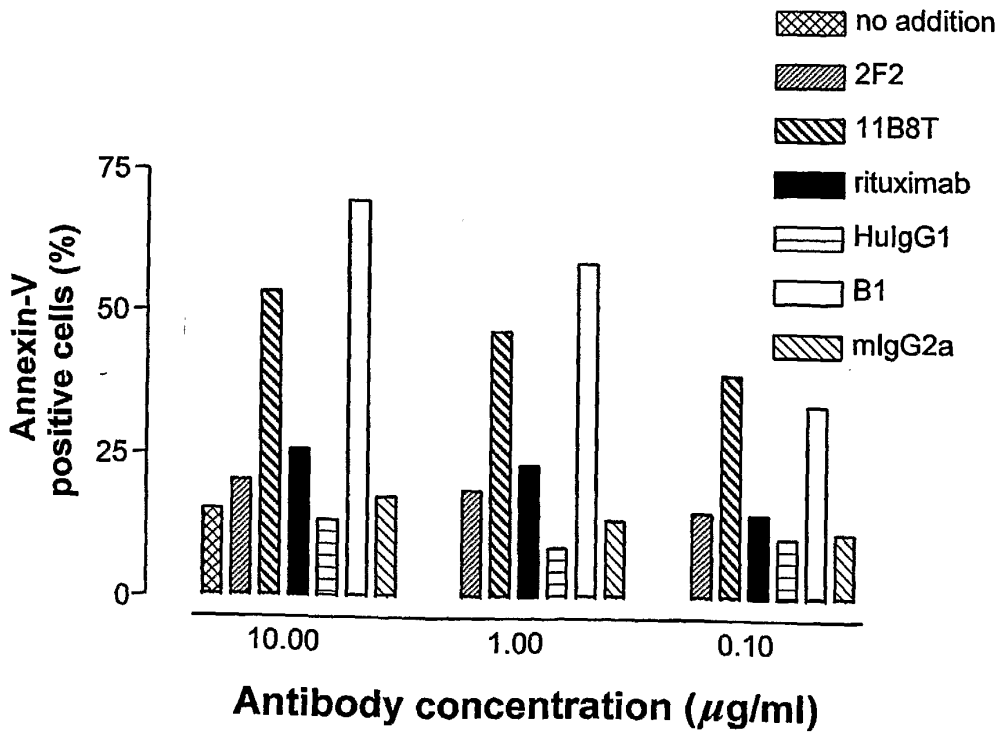


Fig. 34

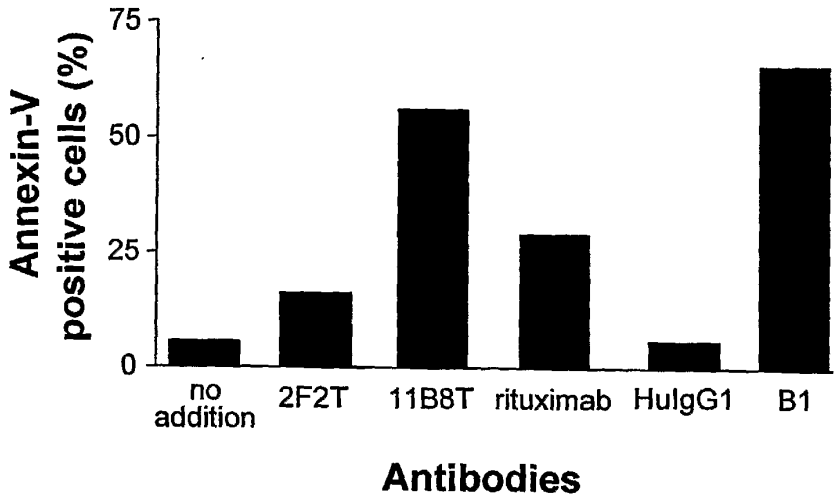

Annexin-V positive cells (%)
75
50
25
0
no addition
2F2T
11B8T
rituximab
HuIgG1
B1
Antibodies

Fig. 35A

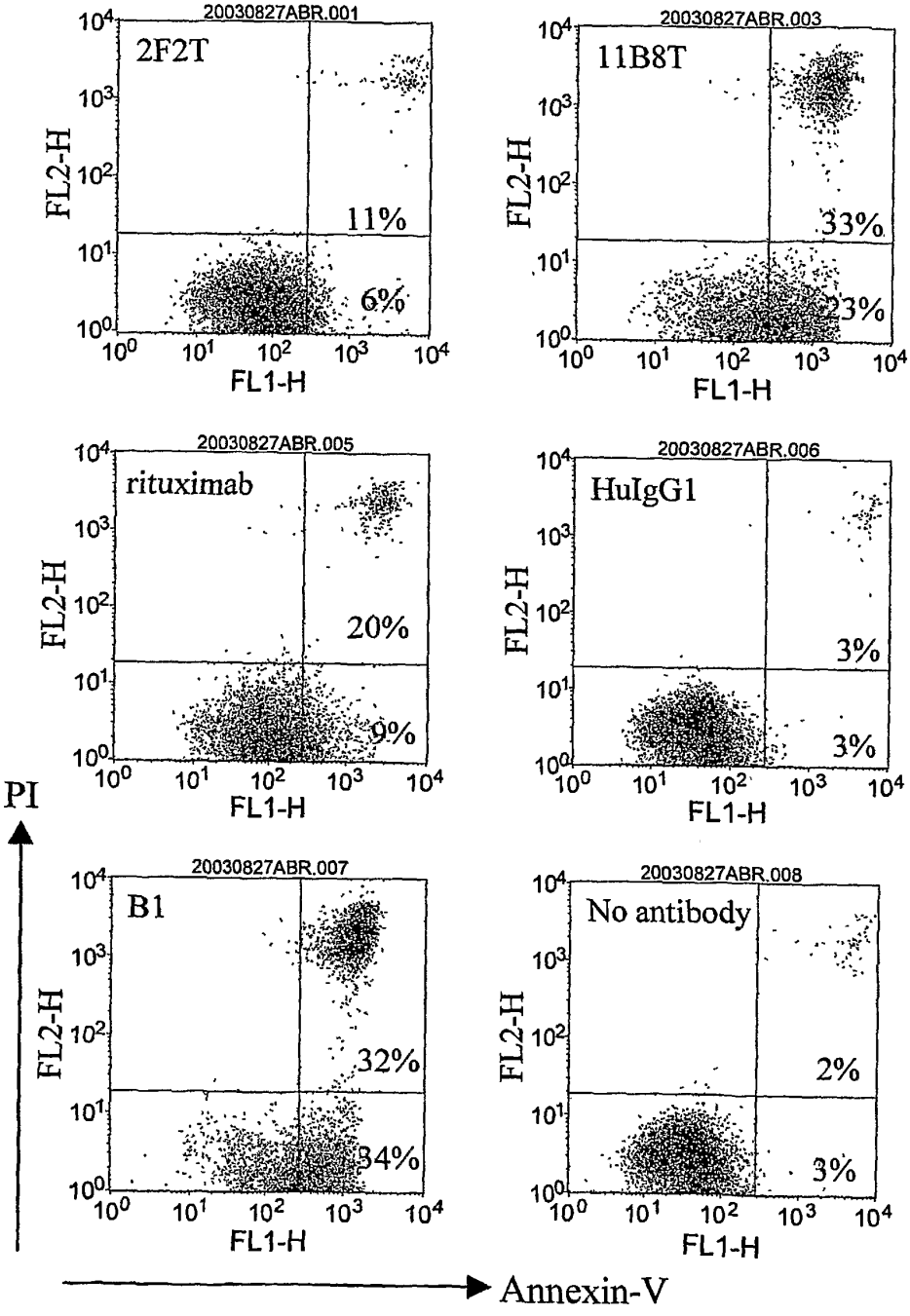


Fig. 35B

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Homotypic adhesion of Ramos cells.

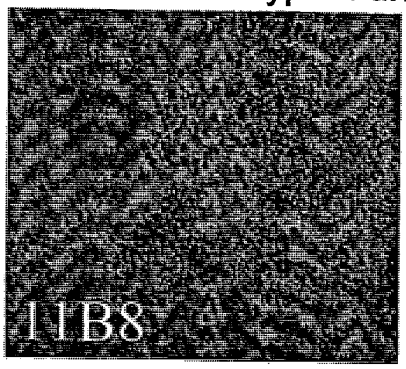


Fig. 36A

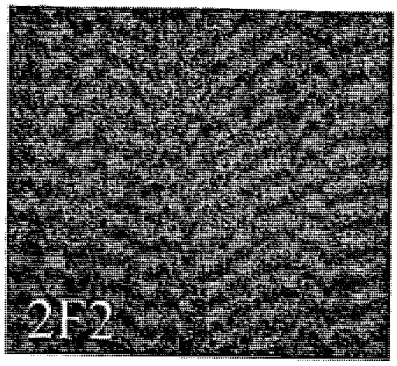


Fig. 36B

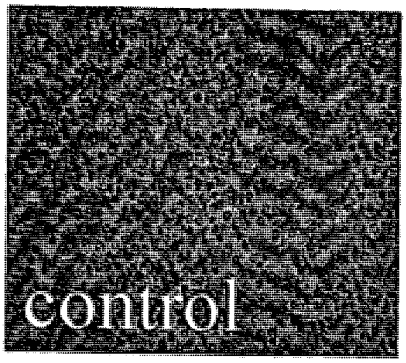


Fig. 36C

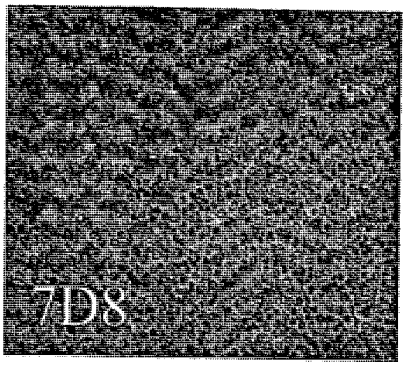


Fig. 36D

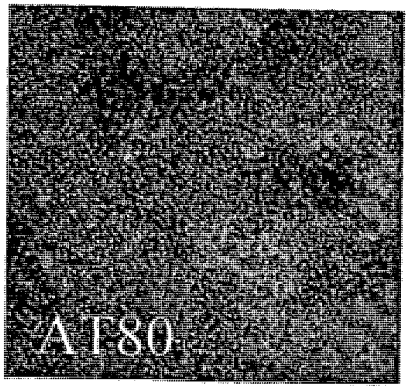


Fig. 36E

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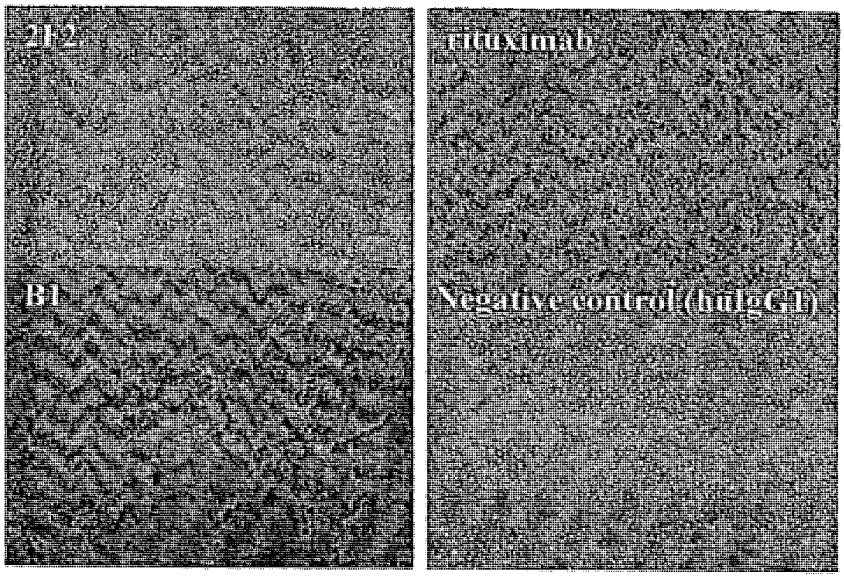


Fig. 37

Samsung Bioepis Exhibit No. 1028
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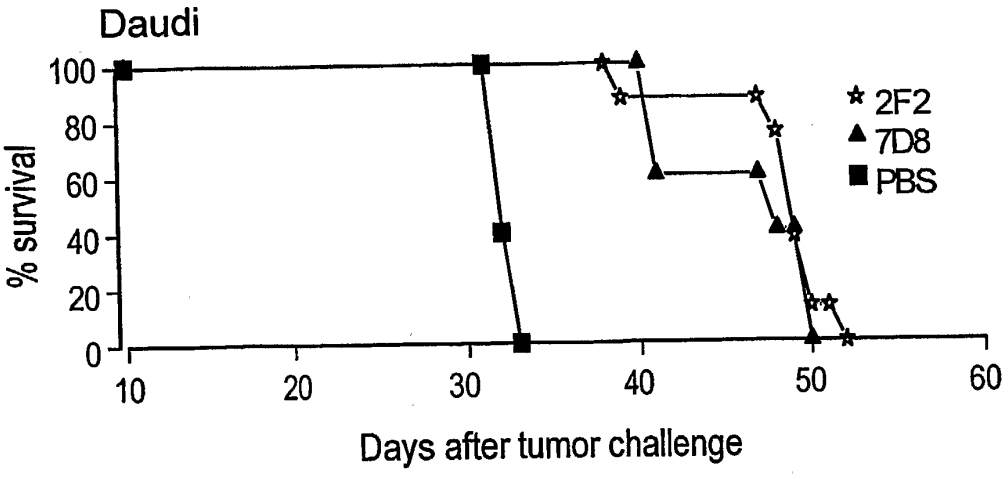


Fig. 38

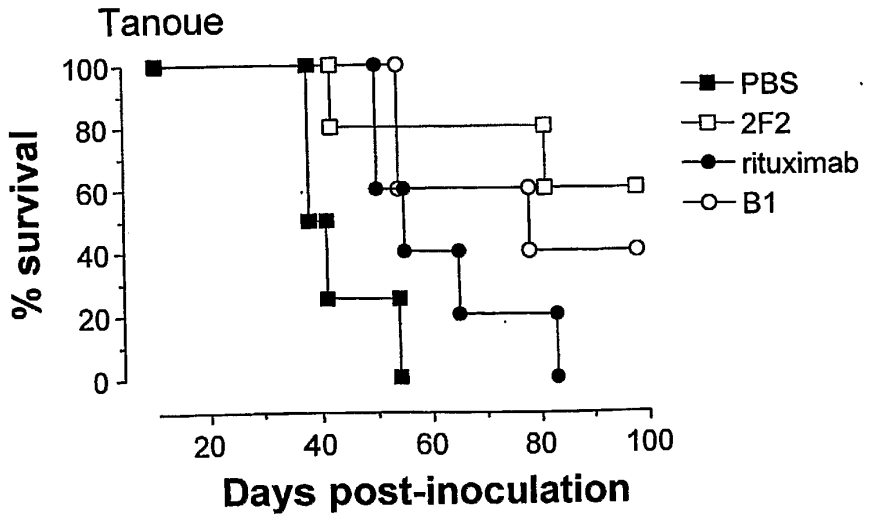


Fig. 39

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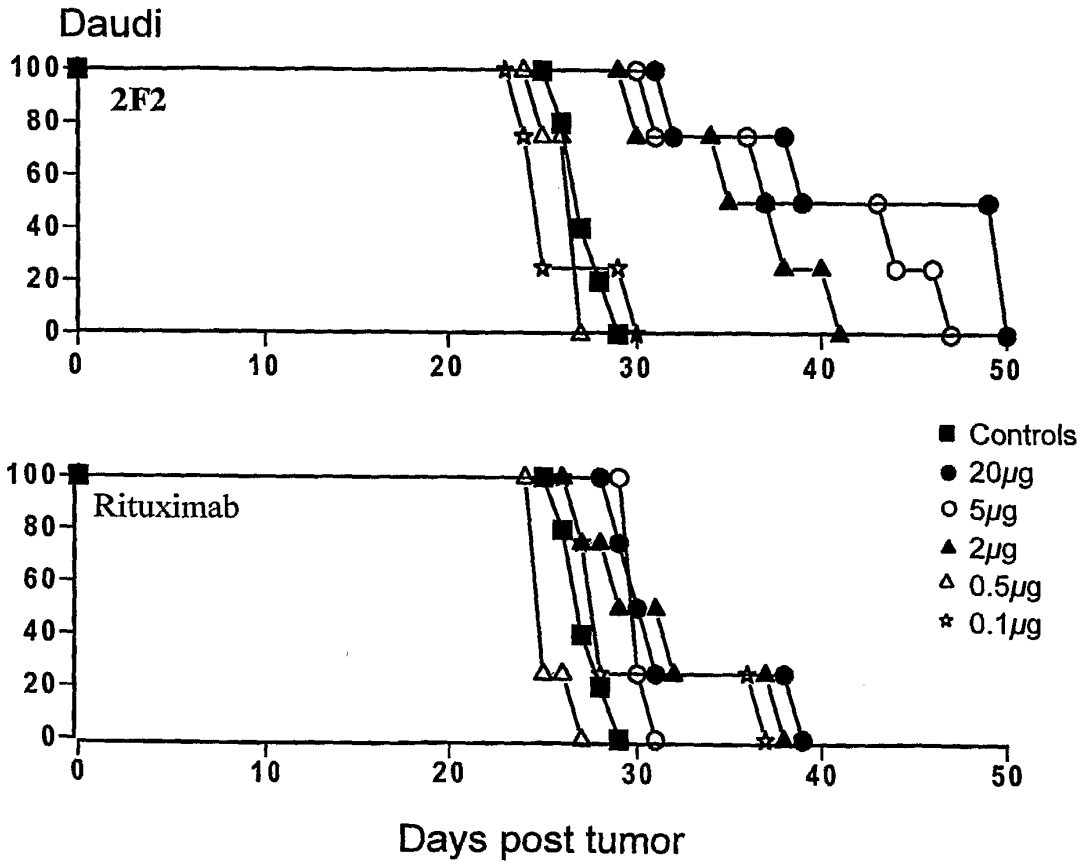

Daudi
2F2
Rituximab
Controls
20μg
5μg
2μg
0.5μg
0.1μg
Days post tumor

Fig. 40

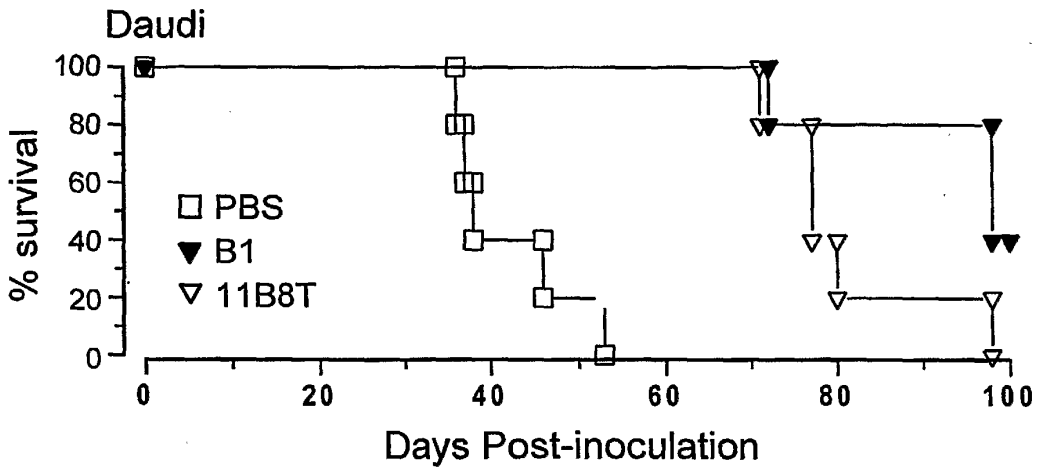

Daudi
100
80
60
40
20
0
% survival
PBS
B1
11B8T
0
20
40
60
80
100
Days Post-inoculation

Fig. 41

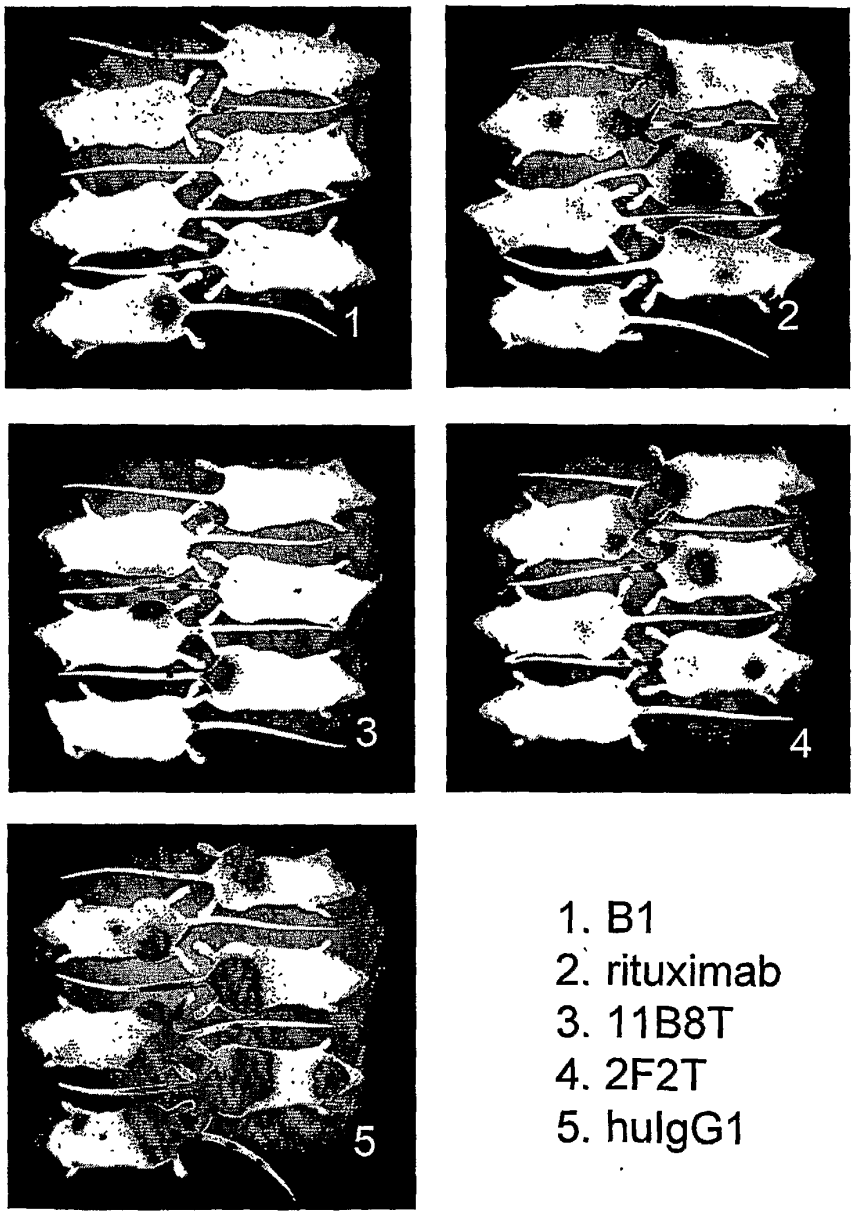


Fig. 42

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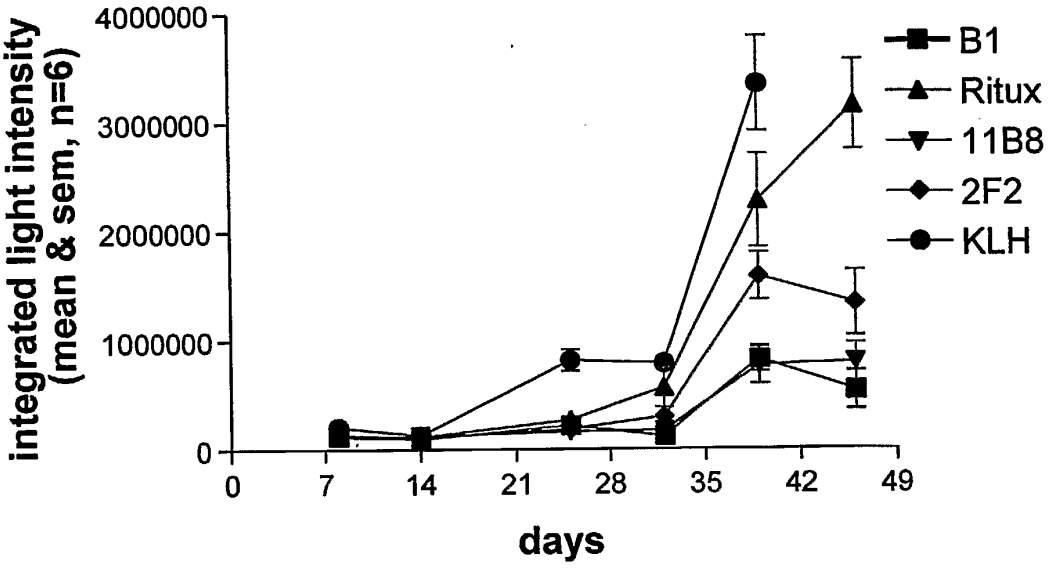

integrated light intensity
(mean & sem, n=6)
4000000
3000000
2000000
1000000
0
0
7
14
21
28
35
42
49
days
B1
Ritux
11B8
2F2
KLH

Fig. 43

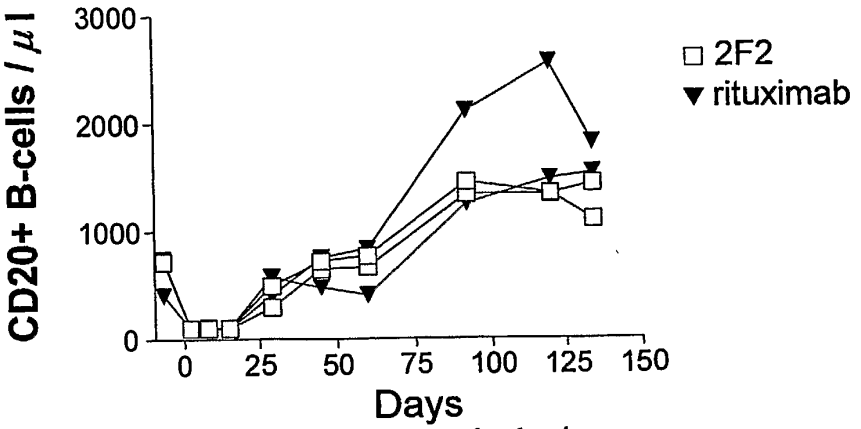


Fig. 44A

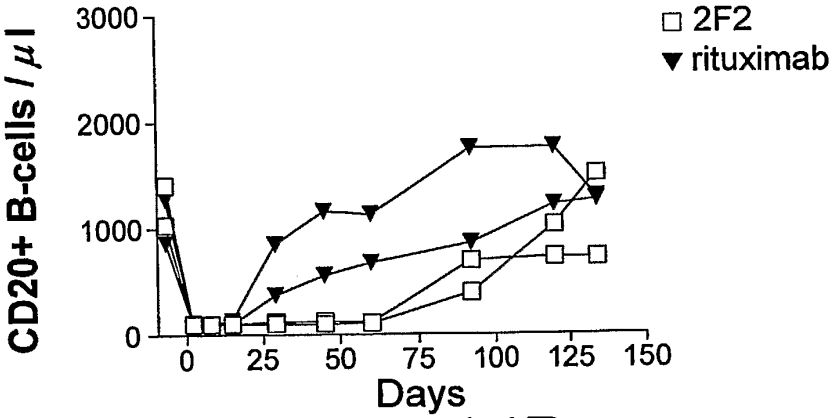


Fig. 44B

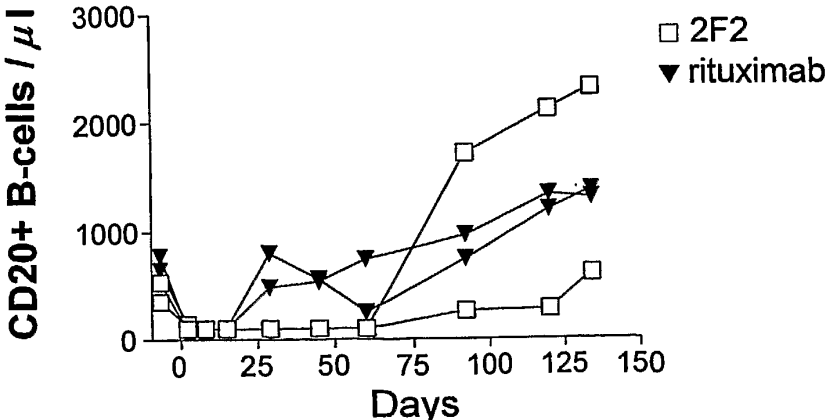


Fig. 44C

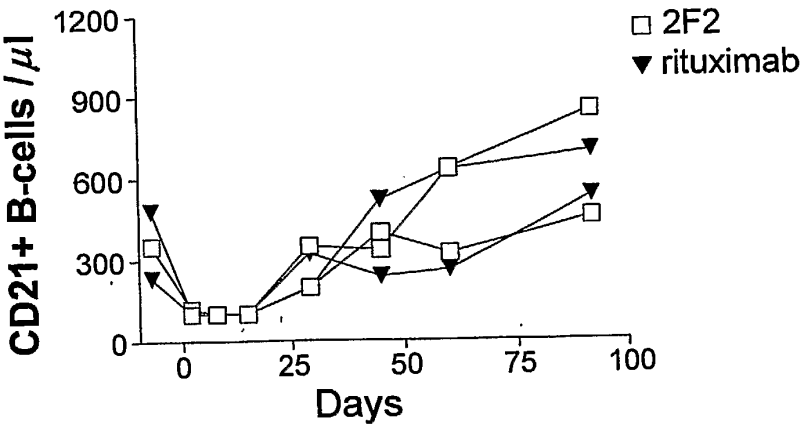


Fig. 45A

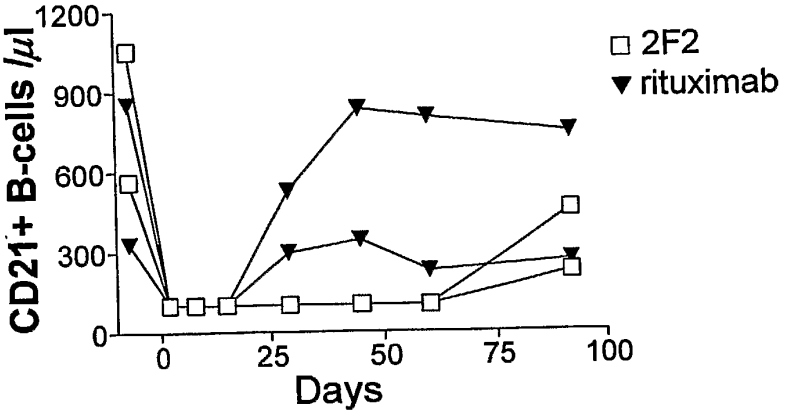


Fig. 45B

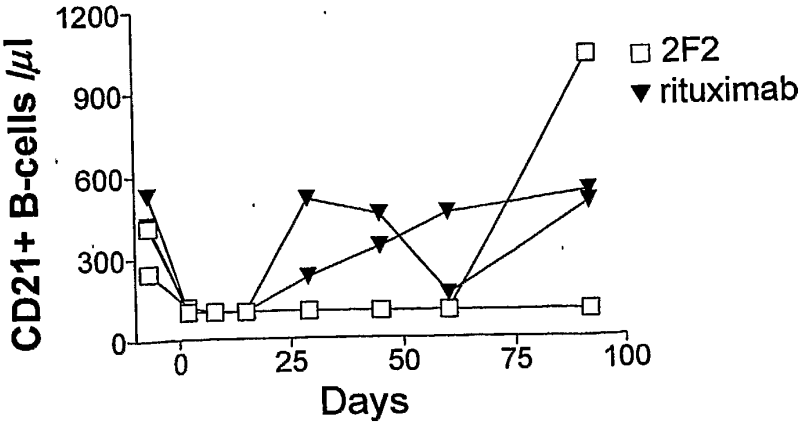


Fig. 45C

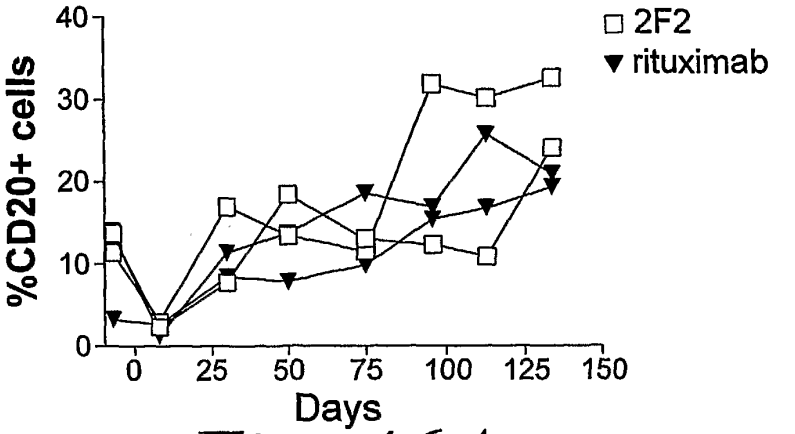


Fig. 46A

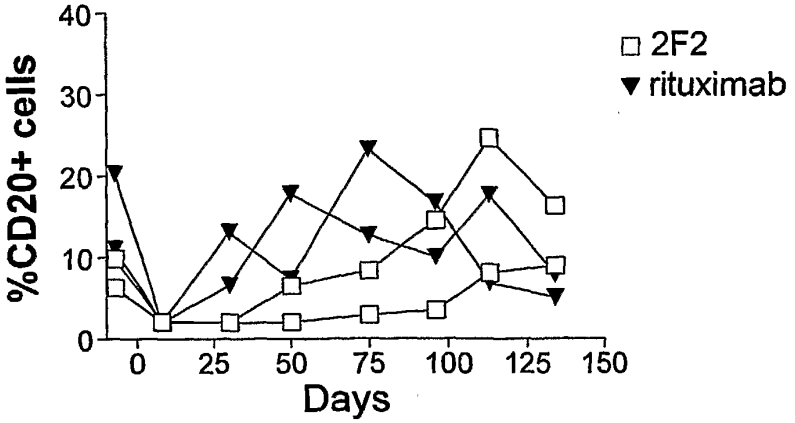


Fig. 46B

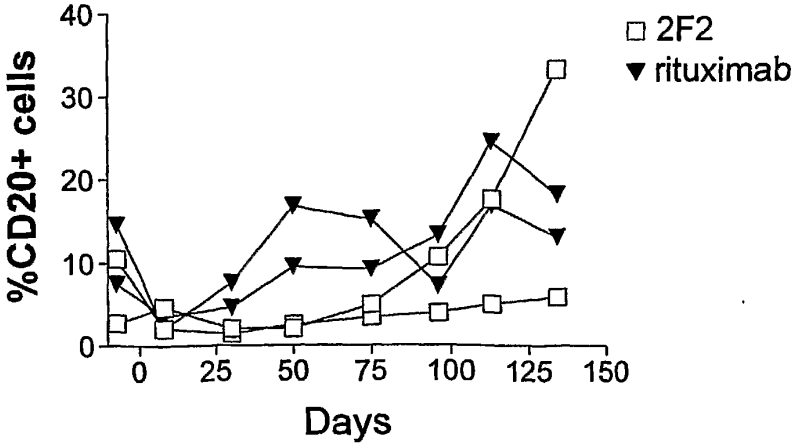


Fig. 46C

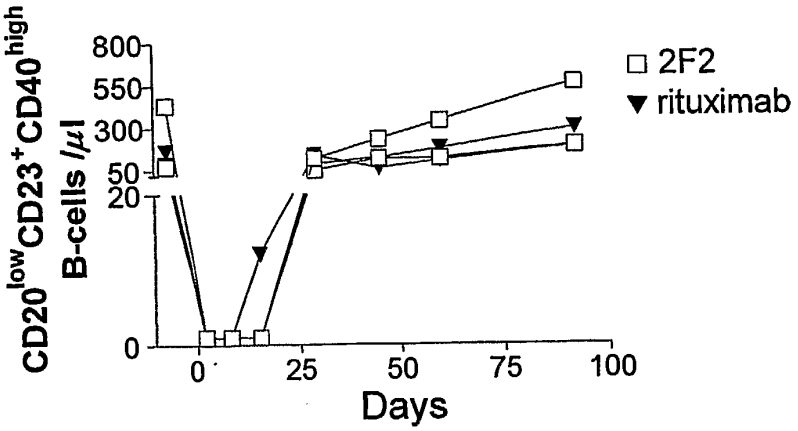


Fig. 47B

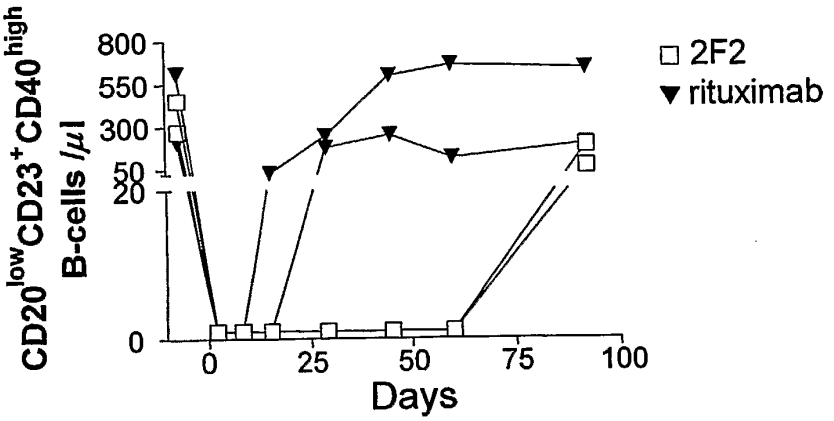


Fig. 47B

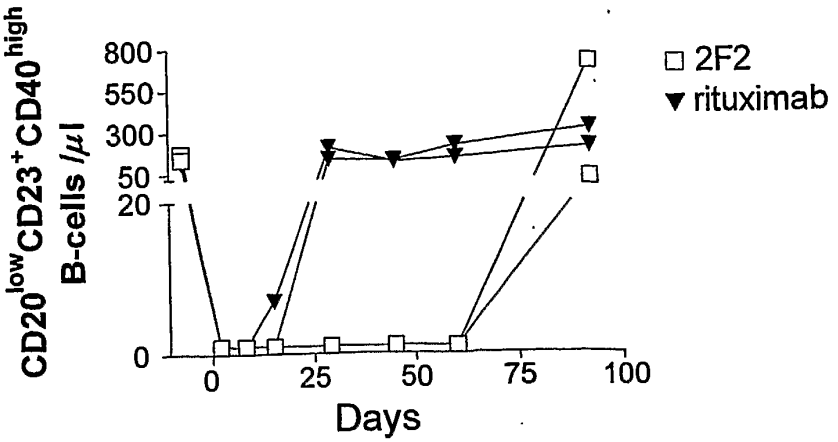


Fig. 47C

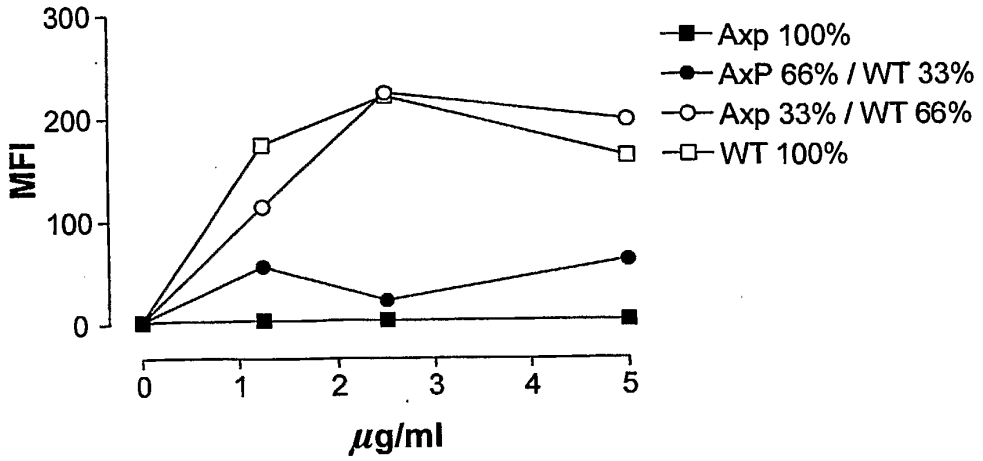


Fig. 48A

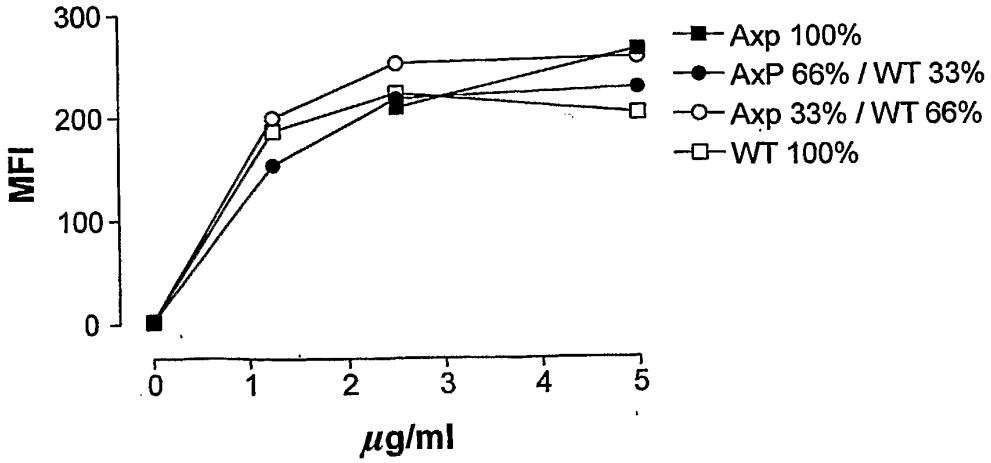


Fig. 48B

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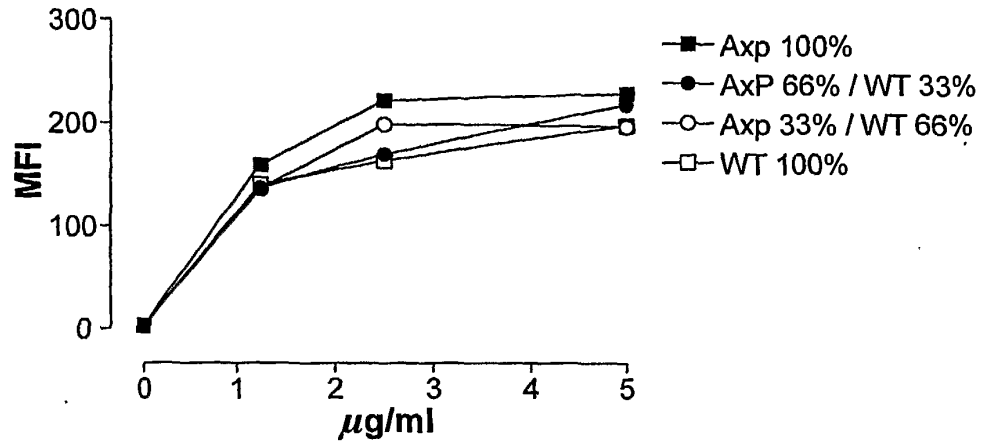


Fig. 48C

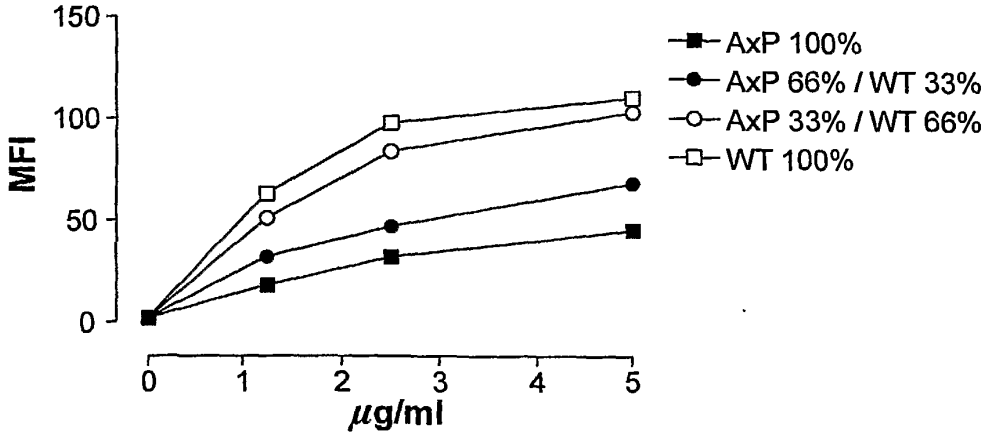


Fig. 48D

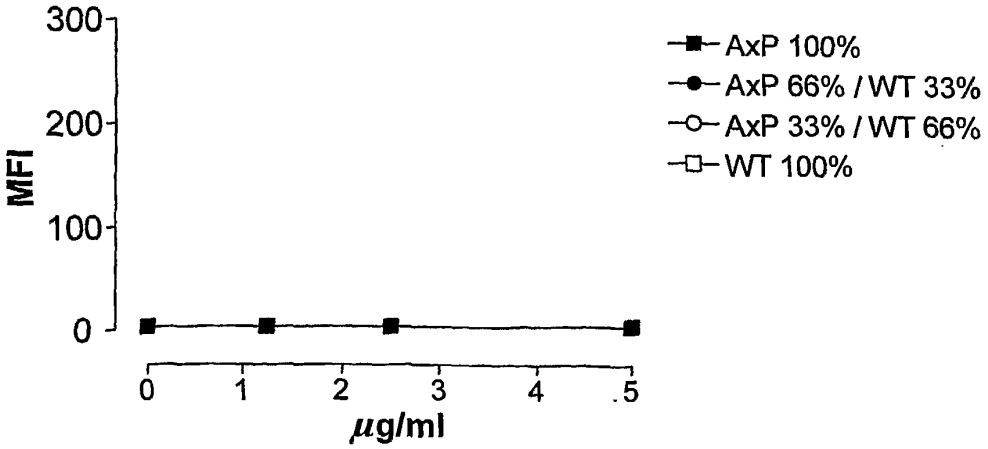


Fig. 48E

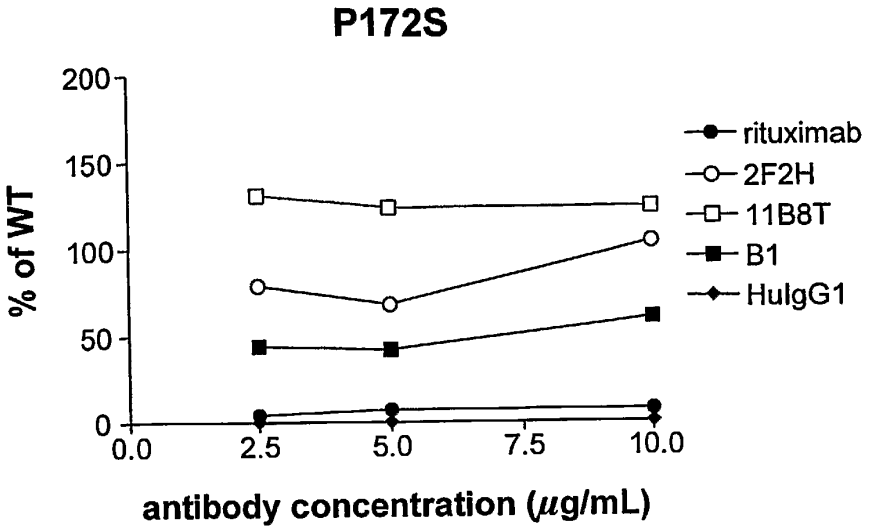


Fig. 49A

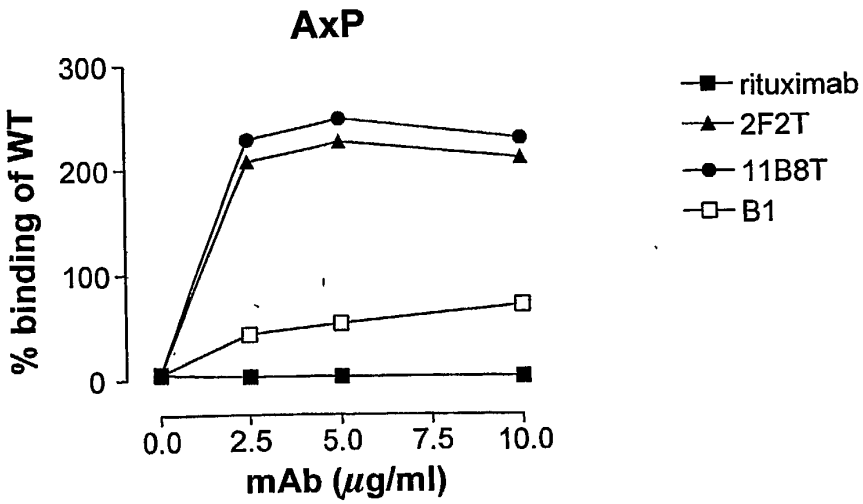


Fig. 49B

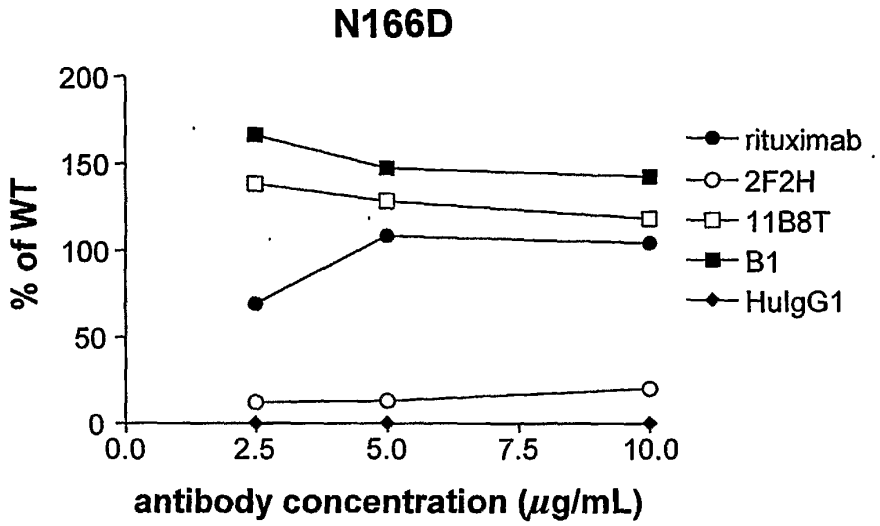


Fig. 49C

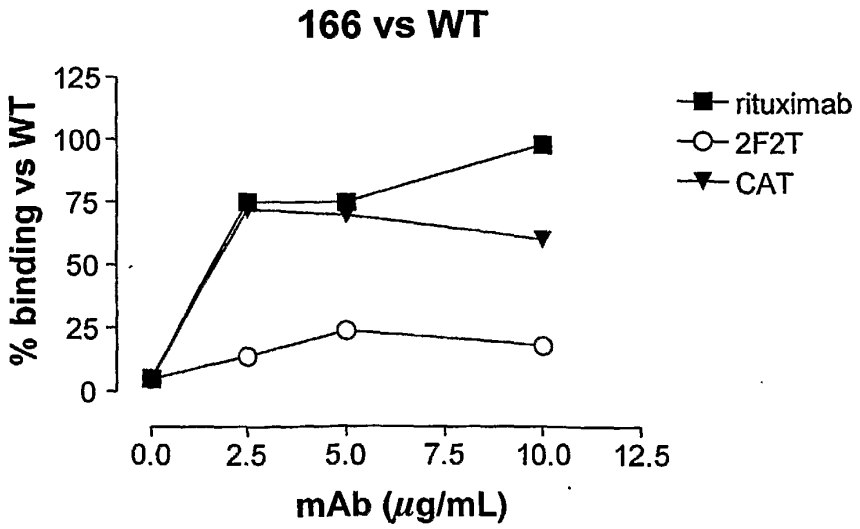


Fig. 49D

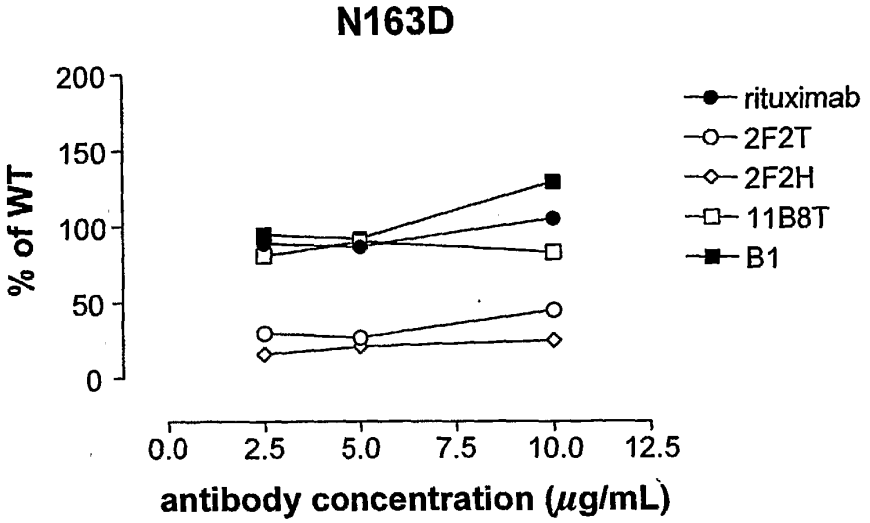


Fig. 49E

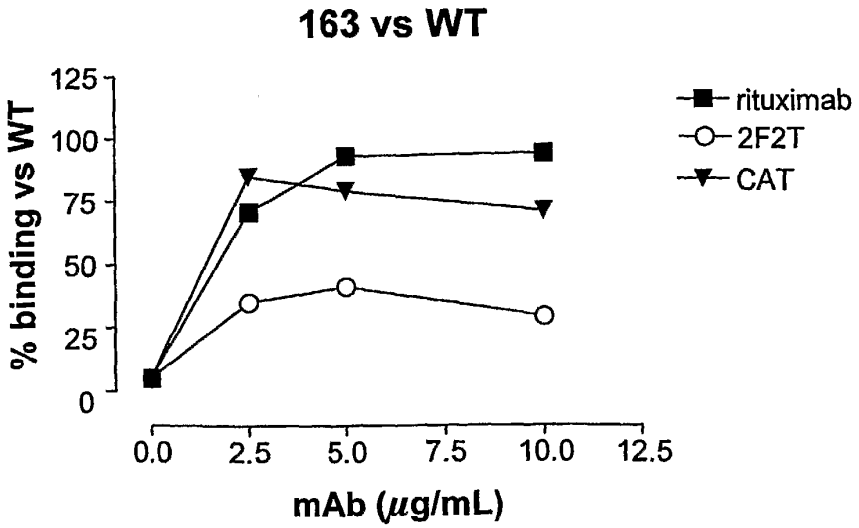


Fig. 49F

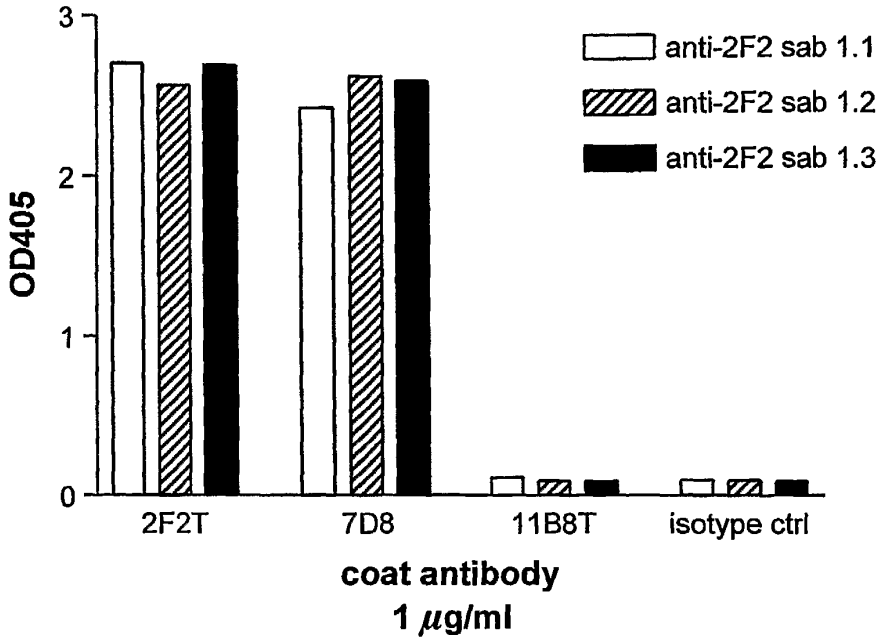


Fig. 50

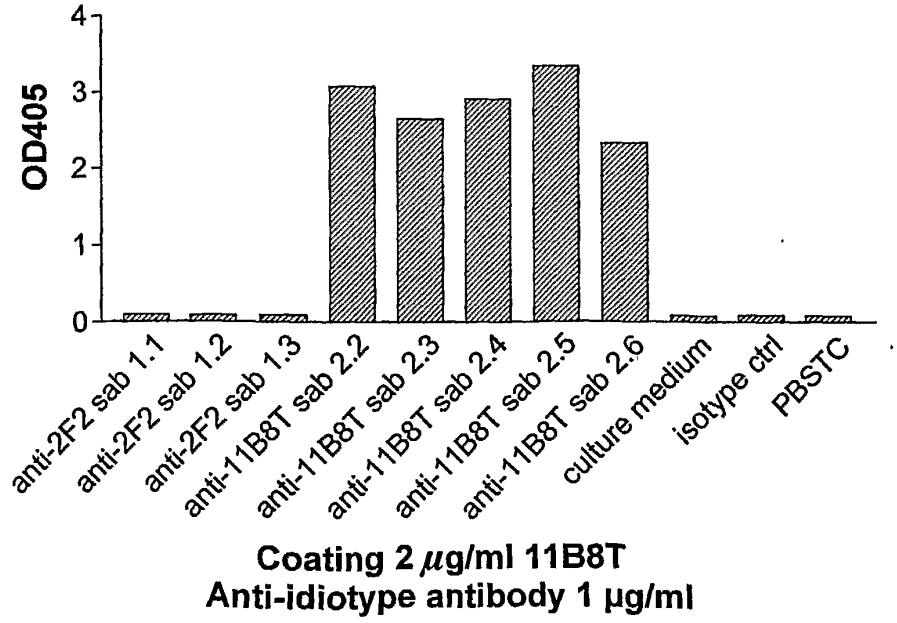


Fig. 51

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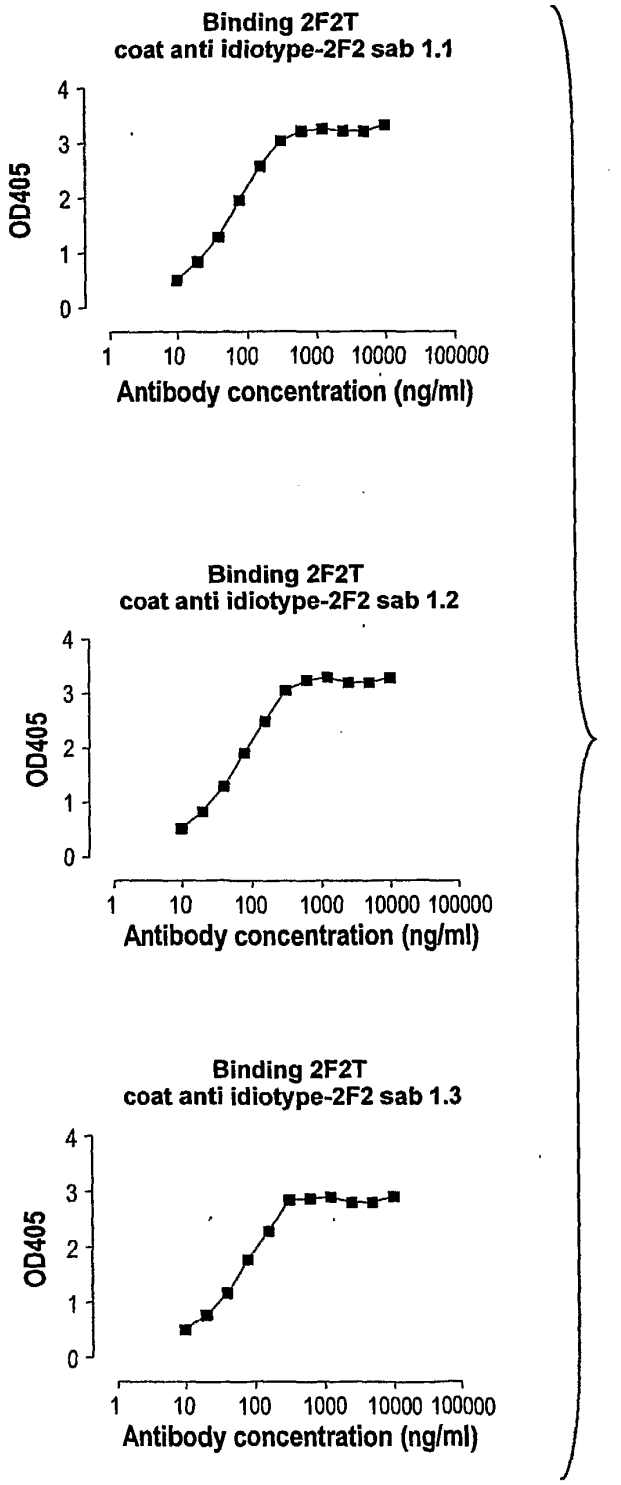


Fig. 52

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Translation of 2F2 VH

```
  1 MFLGLSWIFL LAILKGVQCE VQLVESGGGL VQPGRSLRLS CAASGFTFND
 51 YAMHWVRQAP GKGLEWVSTI SWNSGSIGYA DSVKGRFTIS RDNAKKSLYL
101 QMNSLRAEDT ALYYCAKDIQ YGNYYYGMDV WGQGTTVTVS S
```

Translation of 2F2VL

```
  1 MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51 SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101 EDFAVYYCQQ RSNWPITFGQ GTRLEIK
```

CDR1

CDR2

CDR3

Fig. 53

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2F2 VH

```
1   ATGGAG TTGGGA CTGAGC TGGATT TTCCTT TTGGCT ATTTTA AAAGGT GTCCAG
55  TGTGAA GTGCAG CTGGTG GAGTCT GGGGGA GGCTTG GTACAG CCTGGC AGGTCC
109 CTGAGA CTCTCC TGTGCA GCCTCT GGATTC ACCTTT AATGAT TATGCC ATGCAC
163 TGGGTC CGGCAA GCTCCA GGGAAG GGCCTG GAGTGG GTCTCA ACTATT AGTTGG
217 AATAGT GGTTCC ATAGGC TATGCG GACTCT GTGAAG GGCCGA TTCACC ATCTCC
271 AGAGAC AACGCC AAGAAG TCCCTG TATCTG CAAATG AACAGT CTGAGA GCTGAG
325 GACACG GCCTTG TATTAC TGTGCA AAAGAT ATACAG TACGGC AACTAC TACTAC
379 GGTATG GACGTC TGGGGC CAAGGG ACCACG GTCACC GTCTCC TCAG
```

2F2VL

```
1   ATGGAA GCCCCA GCTCAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
109 GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
163 TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
217 AACAGG GCCACT GGCATC CCAGCC AGGTTC CGTGGC AGTGGG TCTGGG ACAGAC
271 TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
325 CAGCAG CGTAGC AACTGG CCGATC ACCTTC GGCCAA GGGACA CGACTG GAGATT
379 AAAC
```

Fig. 54

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Translation of 7D8VH

```
  1   MELGLSWIFL LAILKGVQCE VQLVESGGGL VQPDRSLRLS CAASGFTFHD
 51   YAMHWVRQAP GKGLEWVSTI SWNSGTIGYA DSVKGRFTIS RDNAKNSLYL
101   QMNSLRAEDT ALYYCAKDIQ YGNYYYGMDV WGQGTTVTVS S
```

Translation of 7D8VL

```
  1   MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51   SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101   EDFAVYYCQQ RSNWPTTFGQ GTRLEIK
```

CDR1

CDR2

CDR3

Fig. 55

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7D8VH

```
   1  ATGGAG TTGGGA CTGAGC TGGATT TTCCTT TTGGCT ATTTTA AAAGGT GTCCAG
  55  TGTGAA GTGCAG CTGGTG GAGTCT GGGGGA GGCTTG GTACAG CCTGAC AGGTCC
 109  CTGAGA CTCTCC TGTGCA GCCTCT GGATTC ACCTTT CATGAT TATGCC ATGCAC
 163  TGGGTC CGGCAA GCTCCA GGGAAG GGCCTG GAGTGG GTCTCA ACTATT AGTTGG
 217  AATAGT GGTACC ATAGGC TATGCG GACTCT GTGAAG GGCCGA TTCACC ATCTCC
 271  AGAGAC AACGCC AAGAAC TCCCTG TATCTG CAAATG AACAGT CTGAGA GCTGAG
 325  GACACG GCCTTG TATTAC TGTGCA AAAGAT ATACAG TACGGC AACTAC TACTAC
 379  GGTATG GACGTC TGGGGC CAAGGG ACCACG GTCACC GTCTCC TCAG
```

7D8VH

```
   1  ATGGAA GCCCCA GCTCAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
  55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
 109  GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
 163  TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
 217  AACAGG GCCACT GGCATC CCAGCC AGGTTC AGTGGC AGTGGG TCTGGG ACAGAC
 271  TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
 325  CAGCAG CGTAGC AACTGG CCGATC ACCTTC GGCCAA GGGACA CGACTG GAGATT
 379  AAAC
```

Fig. 56

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Translation of VHCD2011B8

```
  1   MELGLSWVFL VAILKGVQCE VQLVQSGGGL VHPGGSLRLS CTGSGFTFSY
 51   HAMHWVRQAP GKGLEWVSII GTGGVTYYAD SVKGRFTISR DNVKNSLYLQ
101   MNSLRAEDMA VYYCARDYYG AGSFYDGIYG MDYWGQGTTV TVSS
```

Translation of VLCD2011B8

```
  1   MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51   SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101   EDFAVYYCQQ RSDWPLTFGG GTKVEIK
```

CDR1

CDR2

CDR3

Fig. 57

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VHCD2011B8

```
   1  ATGGAG TTGGGG CTGAGC TGGGTT TTCCTT GTTGCT ATATTA AAAGGT GTCCAG
  55  TGTGAG GTTCAG CTGGTG CAGTCT GGGGGA GGCTTG GTACAT CCTGGG GGGTCC
 109  CTGAGA CTCTCC TGTACA GGCTCT GGATTC ACCTTC AGTTAC CATGCT ATGCAT
 163  TGGGTT CGCCAG GCTCCA GGAAAA GGTCTG GAATGG GTATCA ATTATT GGGACT
 217  GGTGGT GTCACA TACTAT GCAGAC TCCGTG AAGGGC CGATTC ACCATC TCCAGA
 271  GACAAT GTCAAG AACTCC TTGTAT CTTCAA ATGAAC AGCCTG AGAGCC GAGGAC
 325  ATGGCT GTGTAT TACTGT GCAAGA GATTAC TATGGT GCGGGG AGTTTT TATGAC
 379  GGCCTC TACGGT ATGGAC GTCTGG GGCCAA GGGACC ACGGTC ACCGTC TCCTCA
 433  G
```

VLCD2011B8

```
   1  ATGGAA GCCCCA GCACAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
  55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
 109  GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
 163  TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
 217  AACAGG GCCACT GGCATC CCAGCC AGGTTC AGTGGC AGTGGG TCTGGG ACAGAC
 271  TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
 325  CAGCAG CGTAGC GACTGG CCGCTC ACTTTC GGCGGA GGGACC AAGGTG GAGATC
 379  AAAC
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Fig. 58

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GMI-055PC

SEQUENCE LISTING

<110> Genmab, Inc. *et al.*

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<130> GMI-055PC

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<151> 2002-10-17

<150> US 60/460,028
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tgtgcagcct ctggattcac ctttaatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggttccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaagtc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
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tcag 424

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<212> PRT
<213> Homo sapiens

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 20 25 30
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 35 40 45
Asn Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 50 55 60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Ser Ile Gly Tyr Ala
65 70 75 80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys
 85 90 95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
 100 105 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
 115 120 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
 130 135 140

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<212> DNA
<213> Homo sapiens

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
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Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
 35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
 50 55 60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65 70 75 80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
 85 90 95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
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<212> DNA
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caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
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20 25 30
Pro Asp Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50 55 60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65 70 75 80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
85 90 95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
100 105 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
115 120 125
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130 135 140

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ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
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Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
50 55 60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65 70 75 80

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```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
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            20                  25                  30
Pro Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly Ser Gly Phe Thr Phe
        35                  40                  45
Ser Tyr His Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp
65                  70                  75                  80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Val Lys Asn Ser
                85                  90                  95
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr
            100                 105                 110
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        115                 120                 125
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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcgact ggccgctcac tttcggcgga 360
gggaccaagg tggagatcaa ac 382

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<212> PRT
<213> Homo sapiens

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1 5 10 15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
20 25 30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
50 55 60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65 70 75 80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
85 90 95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
100 105 110
Asp Trp Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
115 120 125

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<210> 15
<211> 13
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Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met Asp Val
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<210> 16
<211> 11
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Arg Ala Ser Gln Ser Val Ser Ser Tyr Leu Ala
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1 5

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<210> 20
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<210> 21
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<400> 23
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1 5

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1 5

<210> 25
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<400> 25
Ser Tyr His Ala Met His
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<210> 26
<211> 16
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<400> 26
Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp Ser Val Lys Gly
1 5 10 15

<210> 27
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<400> 27
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1 5 10 15
Val

<210> 28
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<210> 31
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Page 787

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<210> 37
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<220>
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<400> 37
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<210> 38
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<210> 52
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<220>
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20 25 30
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35 40 45
Ser Ser Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50 55 60
Glu Trp Val Ser Ala Ile Gly Thr Gly Gly Gly Thr Tyr Tyr Ala Asp
65 70 75 80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser
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Tyr Cys Ala Arg
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1 5 10 15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
20 25 30

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Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
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Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro
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<400> 56

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 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
Asp Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Gly Ile Ser Trp Asn Ser Gly Ser Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Asp Tyr Tyr Tyr Tyr Tyr Tyr Tyr Gly Met
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    130                 135                 140
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            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
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GMI-055PC

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Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
```

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

15 February 2016

Dear Sirs

European Patent Application No. 11792564.4-1412
KYOWA HAKKO KIRIN CO., LTD. *et al.*
Our Ref: N118545-EP PCT/cxe

In response to the Communication pursuant to Article 94(3) EPC dated 8 October 2015, please amend this application by replacing the claims presently on file with the new claims provided herewith. For the Examiner's assistance a track-changes copy of the claims showing the amendments being made to them is also provided.

Please also replace the sequence listing currently on file with the corrected sequence listing provided herewith. A hand-amended copy of original sequence listing pages 3 to 9 is also attached to show the corrections being made.

A copy of the pages of Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) showing the signal peptides of human kappa light chains and the signal peptides of human heavy chains is attached as evidence of the common general knowledge at the priority date.

An Experimental Report as referred to below is attached.

I am also filing an English translation of the priority document in view of the reference made below to D11, which was cited in categories X and P in the extended European Search Report.

Amendments

Claims 1, 3 and 5 have been amended to specify that the antibody exhibits ADCC activity. This amendment finds basis in the originally filed application at, for example, page 3 lines 11 to 14.

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Inventive Step

The claimed antibodies involve an inventive step. As disclosed at page 3, paragraph 12 of the application, an object of the present invention is to provide a monoclonal antibody or an antibody fragment which binds to the amino acid sequence or the three-dimensional structure of the extracellular region of TIM-3 and expresses ADCC activity.

None of the cited documents disclose an anti-TIM-3 monoclonal antibody having ADCC activity. The present inventors have made the unexpected finding that antibodies binding to a particular epitope in the extracellular domain of TIM-3 have good ADCC activity, whilst antibodies binding to other epitopes do not. The ADCC activity of the antibodies of the invention is therefore linked to their binding to a particular epitope in TIM-3.

As demonstrated in the present application, the anti-TIM-3 monoclonal antibodies produced in Examples 12 to 14 (i.e., antibodies 512, 644, 4545 and 4177) and the anti-TIM-3 monoclonal antibody 8213 bind to IgV domain in the extracellular domain of TIM-3 (see paragraph [0405] of the present application). Further, the identification by the present inventors of the specific binding site in the IgV domain revealed that the antibodies 4545, 4177, and 8213 which bind to the amino acid sequence at positions 67 to 105 of the IgV domain (see paragraph [0425] and [0426] of the present application) have ADCC activity (see paragraphs [0371] and [0375] of the present application).

As explained hereinafter, the ADCC activity exhibited by binding of antibodies 4545 and 4177 of the present invention to the extracellular domain of TIM-3 is dependent on the epitope in TIM-3 and not on the binding activities of these antibodies to the antigen.

The Figure in the attached Experimental Report demonstrates that the anti-TIM-3 monoclonal antibodies 512, 644, 4545, and 4177 have almost equivalent binding activities to TIM-3 expressing HEK293 cells.

Further, the Table in the attached Experimental Report shows that an anti-dinitrophenol (DNP) antibody which was used as negative control exhibited almost no ADCC activity to Daudi cells, while the anti-TIM-3 monoclonal antibodies 4545 and 4177 exhibited significantly higher ADCC activity (see also paragraphs [0371] and [0375] of the present application). On the other hand, the anti-TIM-3 monoclonal antibodies 512 and 644 which bind to different epitopes from the antibodies 4545 and 4177 exhibited almost no ADCC activity.

The results shown in the attached Experimental Report reveal that although the anti-TIM-3 monoclonal antibodies 512, 644, 4545, and 4177 have almost equivalent binding activities to TIM-3, the strength of ADCC activity is different between the antibodies 4545 and 4177 versus the antibodies 512 and 644. This difference in the strength of ADCC activity is attributable to the binding of the 4545 and 4177 antibodies to a different epitope to those of the 512 and 644 antibodies.

Further, Kikushige *et al.* cited as D11 in the extended European Search Report shows that the mouse monoclonal antibody 8213 ("ATIK2a") specifically binds to TIM-3 expressing cells and has ADCC activity (see Figures 5A and 5B of D11). Therefore, the antibodies 4545, 4177 and 8213 of the present invention bind to the specific epitopes in the extracellular domain of TIM-3 and exhibit ADCC activity.

D1 teaches that based on the expression or the function of TIM-3 molecule, an agent affecting TIM-3 activity (including an anti-TIM-3 antibody) may be an antagonist antibody that blocks the binding interactions between TIM-3 and TIM-3 ligands or an agonist antibody that triggers TIM-3 intracellular signaling (see WO08/060617, "C. antibodies" beginning on page 35, first and second paragraphs; and "D. Method to determine effect on TIM-3 activity" beginning on page 38, first and second paragraphs). D1 also teaches that the antibody may bind to the IgV domain in the extracellular domain of TIM-3 (claim 10 of D1). However, D1 does not teach or suggest that ADCC activity of an antibody binding TIM-3 may be a useful property. Nor is there anything in D1 to indicate that ADCC activity of an anti-TIM-3 antibody may depend on the epitope to which it binds.

D2 discloses the specific anti-TIM-3 antibodies 8B.2C12 and 25F.1D6 which were obtained by immunizing rats (WO 03/063792, page 3, lines 20-22; and Example 1). Like D1, D2 does not teach or suggest that ADCC activity of an antibody binding TIM-3 may be a useful property or indicate that ADCC activity of an anti-TIM-3 antibody may depend on the epitope to which it binds.

D3 teaches that the agent can be a chimeric polypeptide that includes a TIM polypeptide and a heterologous polypeptide such as the Fc region of the IgG antibody (WO 2005/097211, page 19, lines 16-19) and the Fc region is defined on page 20, first full paragraph. Further, D3 teaches that TIM-3 is expressed in lymphomas, thymomas, and mastocytomas (page 34, last paragraph; and Example XV). The Examiner considers that D3 contemplates that anti-TIM-3 antibodies may have ADCC activity. However, D3 does not describe ADCC activity of an anti-TIM-3 antibody. Therefore, the skilled person reading D3 would not know that ADCC activity of an antibody which binds to TIM-3 may depend on the specific region (epitope) in the extracellular (or IgV) domain of TIM-3 to which the antibody binds.

None of the cited prior art documents disclose an antibody binding to the same epitope on TIM-3 as the antibodies 4545, 4177 and 8213 or an anti-TIM-3 antibody having ADCC activity. Therefore, the objective technical problem in view of any of D1 to D3 may be defined as the provision of anti-TIM-3 antibodies with ADCC activity. This problem is solved by the present claims, which define antibodies binding to the same epitope as antibodies 4545, 4177 and 8213 and which have ADCC activity.

The skilled person would not have been motivated based on any of D1 to D3 to identify antibodies binding to epitopes in the TIM-3 IgV domain which are associated with ADCC activity. The skilled person would not have expected ADCC activity to be dependent on the epitope to which the antibodies bind. Accordingly, the present claims solve the problem of providing anti-TIM-3 antibodies with ADCC activity and the solution provided by the present claims would not have been obvious to the skilled person at the priority date.

Sufficiency

As described in Example 17, paragraphs [0371] and [0375] of the present application, the antibodies of the present invention have significantly high ADCC activity compared both to antibodies binding to different epitopes in the TIM-3 extracellular domain and to a control antibody that exhibited almost no ADCC activity to Daudi cells. Therefore, the antibodies of the present invention specifically bind to TIM-3 expressing cells and remove the same, and it is clear that the present invention is useful for treating the diseases caused by TIM-3 positive cells.

Correction of Sequence Listing

I request correction under Rule 139 EPC of SEQ ID NOs: 8, 10, 18 and 20 to remove the signal sequences that were erroneously included. It is immediately evident that an error has occurred and what the correction should be.

It would be immediately evident that inclusion of the signal sequences in SEQ ID NOs: 8, 10, 18 and 20 was unintentional. Claim 3 of the original application defines an antibody comprising a VH comprising SEQ ID NO: 8 and a VL comprising SEQ ID NO: 10 and an antibody comprising a VH comprising SEQ ID NO: 18 and a VL comprising SEQ ID NO: 20. The signal sequences of VH and VL chains are not present in an antibody because the sequences are cleaved off during antibody production in cells. Therefore, it is clearly an error that the signal sequences are present in each of SEQ ID NOs: 8, 10, 18 and 20.

Using his common general knowledge, the skilled person would have no difficulty determining which of the amino acids shown in each of SEQ ID NOs: 8, 10, 18 and 20 represent signal sequences.

As disclosed at page 67, paragraphs [0222] and [0223] of the present application, the presence of a signal sequence in a VL or VH amino acid sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies. As shown in the reference document attached hereto (Kabat et al., Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)), the signal sequences of SEQ ID NOs: 10 (VL), 18 (VH) and 20 (VL) are the same as those disclosed in Kabat et al., and the signal sequence of SEQ ID NO: 8 (VH) is almost the same as those disclosed in Kabat et al. except for only one residue (i.e., 11th Val is replaced with Ala).

In this regard, the signal sequences derived from known heavy and light chains, which are the same as (or almost the same as) those of SEQ ID NOs: 8, 10, 18 and 20, were the common technical knowledge as of the filing date. Thus, the ordinary skilled person would immediately recognise which parts of SEQ ID NOs: 8, 10, 18 and 20 represent the amino acid sequences of the signal sequences.

Accordingly, the correction of SEQ ID NOs: 8, 10, 18 and 20 to remove the signal sequences meets with the requirements of Rule 139 EPC.

Conclusion

Favourable reconsideration is respectfully requested in view of the amendments now made and the arguments set out above. If, however, there are any queries outstanding, please issue an Examination Report or telephone. Only as a precaution in case the Examiner is minded to refuse the application, I request Oral Proceedings.

In addition, if the Examiner is minded to issue a Rule 71(3) Communication but wishes to propose amendments to the claims or description, it is requested that the Examiner telephones the undersigned to discuss this before the Rule 71(3) Communication is issued. I look forward to hearing from the Examiner in due course.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH

DECLARATION

I, Tom Vink, Associate Director, Cell & Molecular Sciences at Genmab B.V. do hereby declare and state:

1. Since 1 July 2002, I am an employee of Genmab B.V., which is an affiliate of Genmab A/S.

2. I have thorough experience within the fields of molecular biology, protein biochemistry and antibody engineering

3. PCT application No. WO 2004/035607 has been disclosed to me.

4. The application relates to human monoclonal antibodies against CD20, and it exemplifies three antibodies, 2F2, 7D8 and 11B8. The application contains claims relating to human monoclonal anti-CD20 antibodies, wherein the antibodies are characterized by the variable heavy chain (V_H) and variable light chain (V_L) amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12), cf. claims 15, 17 and 19 of the application:

	2F2	**7D8**	**11B8**
V_H nucleotide	SEQ ID NO:1	SEQ ID NO:5	SEQ ID NO:9
V_L nucleotide	SEQ ID NO:3	SEQ ID NO:7	SEQ ID NO:11
V_H amino acid	SEQ ID NO:2	SEQ ID NO:6	SEQ ID NO:10
V_L amino acid	SEQ ID NO:4 (identical to SEQ ID NO:8)	SEQ ID NO:8 (identical to SEQ ID NO:4)	SEQ ID NO:12

5. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the leader sequences erroneously are included in the variable heavy chain and light chain amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12) which define the mature antibodies 2F2, 7D8 and 11B8, respectively, and (ii) in the affirmative, how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

6. For the reasons explained in the following I believe it would have been obvious for a person skilled in the art at the date of the earliest priority date on 17 October 2002 (i) that the leader sequences (marked in red in Appendix A attached hereto) are

included in the variable heavy chain and light chain amino acid sequences, and (ii) how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

7. It is well known that antibodies are secretory proteins, i.e. proteins that are transported to the extracellular medium by passing through the intracellular secretory pathway of the cell, the first compartment of which is the endoplasmic reticulum. Like other secretory proteins, an antibody heavy or light chain protein is initially produced as a precursor polypeptide containing a so called signal or leader peptide (also denoted leader sequence), which is necessary to direct the polypeptide into the endoplasmic reticulum for further transport and secretion. As for other soluble secretory proteins, during the transport into the endoplasmic reticulum, the signal peptides of the antibody heavy and light chains are cleaved off, resulting in a final mature protein (see also page 29, lines 35-36 of the application). In conclusion, any heavy or light chain from an antibody produced by e.g. a hybridoma will be derived from DNA and RNA constructs in which the heavy and light chain encoding sequences are immediately preceded by the leader sequences.

8. To determine the cleavage site between the leader sequence and the antibody sequence so as to determine where the leader sequence stops and where the antibody sequence starts, a comparison can be made with known antibody sequences. Comparison of the protein sequence (precursor polypeptide) containing the leader sequence, as deduced from the cloned RNA sequence, with a database containing known human antibody protein sequences (such as the Vbase as described on page 71, lines 7-8 of the application) will reveal which part of the protein is the signal peptide and where the mature protein starts. At the earliest priority date of the application, several of these databases were available, including the above Vbase as well as the Kabat [1] or IMGT [2] databases.

9. More particularly, these databases contain collections of all germline signal peptides of all human antibodies, and a simple alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with these signal peptide databases would reveal which part of the sequences are the signal peptides and which part of the sequences are the V_H/V_L sequences. Moreover, comparison/alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with the collection of mature human V_H and V_L sequences in these databases would reveal where the mature proteins

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start.

As an example, in Figure 1, a selection of V_H germline leader peptides is shown (screenshot from the Vbase). The leader peptide of V_H 7D8, MELGLSWIFLLAILKGVQC, is easily identified as sequence VH3 3-09.

Alternatively, using the DNA plot module on the Vbase site (which, to my knowledge, was available prior to the earliest priority date of 17 October 2002) it is possible to align the nucleotide sequence of your rearranged V gene to its closest mature germline V. In Figure 2 this was done for the V_L region of 11B8 and, as is shown in the screenshot, the start of the mature V_L encoding sequence is gaaatt, confirming the start of the mature V_L polypeptide sequence at the corresponding amino acids EI. This can also be done with the Vquest module at the IMGT site, see Figure 3.

10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

11. Alternatively, at the earliest priority date of the application several predictive methods were described, such as [3-5], by which signal peptide sequences could be predicted from a protein sequence. Using the SignalP server (which was available prior to the earliest priority date of 17 October 2002) we have performed this for the V_H polypeptide of antibody 7D8 and a screenshot of the result is shown in Figure 4, indicating the signal peptide cleavage site between the C and E amino acid, confirming the start of the mature V_H region as EV.

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12. In conclusion, it is clear that the claims defining the mature antibodies by the variable heavy chain and light chain amino acid sequences by mistake contain the leader sequences and that nothing else was intended than to define the mature antibodies without these leader sequences.

13. 2F2, 7D8 and 11B8 are characterized by having the following V_H CDR1 regions in the application, cf. for example claims 33, 36 and 40 of the application:

	2F2	**7D8**	**11B8**
V_H CDR1	SEQ ID NO:13	SEQ ID NO:19	SEQ ID NO:25

The CDR regions (SEQ ID Nos: 13-18, 19-24, 25-30) are highlighted in the variable heavy and light chain sequences in Appendix B attached hereto.

14. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the V_H CDR1 regions erroneously contain an additional amino acid, and (ii) in the affirmative, how the correct V_H CDR1 regions should read.

15. I believe that it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 that (ii) the V_H CDR1 regions contain an additional amino acid, and (ii) how the correct CDR1 regions should read for the below reasons.

16. At the earliest priority date of the application several different methods were available to determine the CDR regions of antibodies. The most common method was the Kabat method [1] (see also [6] page 432-433 for an comprehensive manual for assigning the CDR regions of an antibody using the Kabat numbering scheme). Analysis of SEQ ID Nos. 14, 15, 16, 17, and 18 (2F2), 20, 21, 22, 23, and 24 (7D8) and 26, 27, 28, 29, 30 (11B8) defining the V_H CDR2 and CDR3 regions and the V_L CDR1, CDR2 and CDR3 regions of 2F2, 7D8 and 11B8, respectively, shows that the Kabat numbering scheme has indeed been used in this application.

17. When applying the Kabat numbering rules to the V_H CDR1, cf. [6] page 432: *"Start; Approximately residue 31 (always 9 after a C (Cys))*

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Residues before; always CXXXXXXXX (X meaning; any amino acid)
Residue after; Always W. Typically WV, but also WI, WA
Length; 5-7 residues "

It is obvious when applying these rules to the V_H CDR1 regions that the respective V_H CDR1 regions should be "Asp Tyr Ala Met His" for SEQ ID No 13, "Asp Tyr Ala Met His" for SEQ ID No 19 and "Tyr His Ala Met His" for SEQ ID No 25, respectively. When comparing these to the V_H CDR1 sequences as defined in the application, it appears that, by mistake, an extra amino acid has been added to these V_H CDR1 regions.

18. In conclusion, in view of the Kabat rules consistently applied in the application to designate the CDR regions, it is clear that the addition of a further amino acid to the V_H CDR1 sequences was a mistake and that nothing else was intended than to define the V_H CDR1 sequences without inclusion of this additional amino acid.

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

6 Aug 2009
Date

Tom Vink

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References

1. Kabat, E.A., et al., *Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242.* Bethesda, MD: US Department of Health and Human Services, 1991.
2. Lefranc, M.P., *IMGT, the international ImMunoGeneTics database.* Nucleic Acids Res, 2001. **29**(1): p. 207-9.
3. Chou, K.C., *Prediction of signal peptides using scaled window.* Peptides, 2001. **22**(12): p. 1973-9.
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5. Nielsen, H., et al., *Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites.* Protein Eng, 1997. **10**(1): p. 1-6.
6. Kontermann, R. and S. Dübel, *Antibody engineering.* 2001: Springer Heidelberg.

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Apendix A

<210> 1 V_H 2F2
<211> 424
<212> DNA
<213> Homo sapiens

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gtgcagctgg tggagtctgg gggaggcttg gtacagcctg gcaggtccct gagactctcc 120
tgtgcagcct ctggattcac ctttaatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggttccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaagtc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag 424

<210> 2 V_H 2F2
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

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Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Asn	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Ser	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

<210> 3 V_L 2F2
<211> 382
<212> DNA
<213> Homo sapiens

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240

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aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac 382

<210> 4 V_L 2F2
<211> 127
<212> PRT
<213> Homo sapiens

<400> 4

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1				5				10						15	
Asp	Thr	Thr	Gly	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser
			20					25					30		
Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser
		35					40					45			
Val	Ser	Ser	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro
	50					55					60				
Arg	Leu	Leu	Ile	Tyr	Asp	Ala	Ser	Asn	Arg	Ala	Thr	Gly	Ile	Pro	Ala
65					70					75					80
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser
				85					90					95	
Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys	Gln	Gln	Arg	Ser
			100					105					110		
Asn	Trp	Pro	Ile	Thr	Phe	Gly	Gln	Gly	Thr	Arg	Leu	Glu	Ile	Lys	
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<210> 5 V_H 7D8
<211> 424
<212> DNA
<213> Homo sapiens

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tgtgcagcct ctggattcac ctttcatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggtaccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaactc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag 424

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<210> 6 V_H 7D8
<211> 141
<212> PRT
<213> Homo sapiens

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            20                  25                  30
Pro Asp Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
        115                 120                 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
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<210> 7 V_L 7D8
<211> 382
<212> DNA
<213> Homo sapiens

<400> 7

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac                                          382
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<210> 8 V_L 7D8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 8

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 1               5                  10                  15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
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<210> 9 V_H 11B8
<211> 433
<212> DNA
<213> Homo sapiens

<400> 9

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tgtacaggct ctggattcac cttcagttac catgctatgc attgggttcg ccaggctcca 180
ggaaaaggtc tggaatgggt atcaattatt gggactggtg gtgtcacata ctatgcagac 240
tccgtgaagg gccgattcac catctccaga gacaatgtca agaactcctt gtatcttcaa 300
atgaacagcc tgagagccga ggacatggct gtgtattact gtgcaagaga ttactatggt 360
gcggggagtt tttatgacgg cctctacggt atggacgtct ggggccaagg gaccacggtc 420
accgtctcct cag                                                    433
```


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<210> 10 V_H 11B8
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

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            20                  25                  30
Pro Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly Ser Gly Phe Thr Phe
        35                  40                  45
Ser Tyr His Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp
65                  70                  75                  80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Val Lys Asn Ser
                85                  90                  95
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr
            100                 105                 110
Tyr Cys Ala Arg Asp Tyr Tyr Gly Ala Gly Ser Phe Tyr Asp Gly Leu
        115                 120                 125
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
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<210> 11 V_L 11B8
<211> 382
<212> DNA
<213> Homo sapiens

<400> 11

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcgact ggccgctcac tttcggcgga 360
gggaccaagg tggagatcaa ac                                          382
```


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<210> 12 V_L 11B8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 12

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1				5					10					15	
Asp	Thr	Thr	Gly	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser
			20					25					30		
Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser
		35					40					45			
Val	Ser	Ser	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro
	50					55					60				
Arg	Leu	Leu	Ile	Tyr	Asp	Ala	Ser	Asn	Arg	Ala	Thr	Gly	Ile	Pro	Ala
65					70					75					80
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser
				85					90					95	
Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys	Gln	Gln	Arg	Ser
			100					105					110		
Asp	Trp	Pro	Leu	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys	
		115					120					125			

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Appendix B

<210> 2 V_H 2F2 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

Met	Glu	Leu	Gly	Leu	Ser	Trp	Ile	Phe	Leu	Leu	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	[illegible]	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Asn	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Ser	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

<210> 6 V_H 7D8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 6

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1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Asp	Arg	Ser	Leu	Arg	Leu	Ser	[illegible]	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
His	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Thr	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

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<210> 10 V_H 11B8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

Met	Glu	Leu	Gly	Leu	Ser	Trp	Val	Phe	Leu	Val	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Leu	Val	His
			20					25					30		
Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Xaa	Thr	Gly	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Ser	Tyr	His	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Ile	Ile	Gly	Thr	Gly	Gly	Val	Thr	Tyr	Tyr	Ala	Asp
65					70					75					80
Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Val	Lys	Asn	Ser
				85					90					95	
Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Met	Ala	Val	Tyr
			100					105					110		
Tyr	Cys	Ala	Arg	Asp	Tyr	Tyr	Gly	Ala	Gly	Ser	Phe	Tyr	Asp	Gly	Leu
		115					120					125			
Tyr	Gly	Met	Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser
	130					135					140				

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Figure 1
A screenshot is shown of part of the germline V_H signal peptides in the Vbase database.

VH Leader - Amino acid sequence alignment

		-19 -10 -1
		| | |
VH1	1-02	MDWIWRILFLVAAATGAHS
	1-03	MDWIWRILFLVAAATGAHS
	1-08	MDWIWRILFLVAAATSAHS
	1-18	MDWIWSILFLVAAPTGAHS
	1-24	MDCIWRILFLVAAATGIHA
	1-45	MDWIWRILFLVAAATDAYS
	1-46	MDWIWRVFCLLAVAPGAHS
	1-58	MDWIWRILFLVGAATGAHS
VH2	2-05	MDILCSTLLLLTIPSWVLS
	2-26	MDILCYTLLLLTTPSWVLS
VH3	3-07	MELGLSWVFLVAILEGVQC
	3-09	MELGLSWIFLLAILKGVQC
	3-11	MEFGLSWVFLVAIIKGVQC
	3-13	MELGLSWVFLVAILEGVQC
	3-15	MEFGLSWIFLAAILKGVQC
	3-20	MEFGLSWVFLVAILKGVQC
	3-21	MELGLRWVFLVAILEGVQC
	3-23	MEFGLSWLFLVAILKGVQC
	3-30	MEFGLSWVFLVALLRGVQC
	3-33	MEFGLSWVFLVALLRGVQC

Figure 2
A screenshot of an analysis performed using the DNAplot module on the Vbase site. The complete DNA sequence of the V_L 11B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

Thank you for using DNAPLOT and V BASE !

Alignment for V segment

EMBL	Locus	Name	score	GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGG
X01668	L6	Vg/38K...+	1305	.....
X17264	L20	Vg^n/13K11...+	1278	.....
L19271	-	3A7	1260	.....
L19272	-	3A9	1242	.....G.....
X93639	A27	DPK22/A27...+	1224	.....G.....G.....

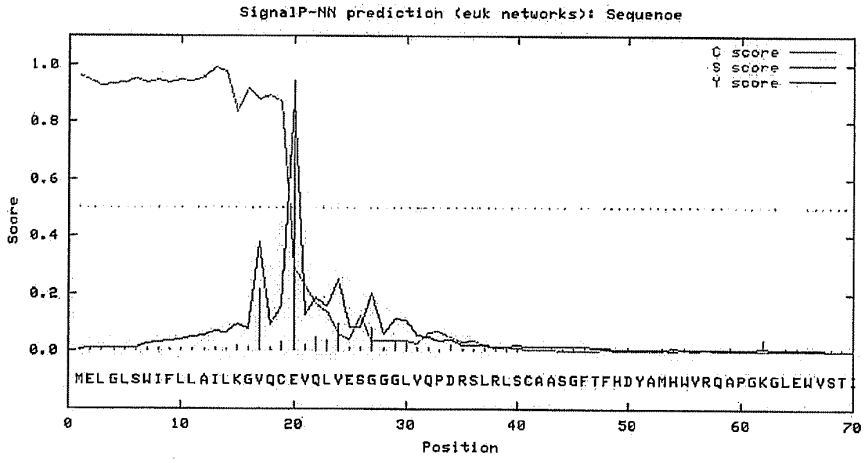
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Figure 3
A screenshot of an analysis performed using the VQUEST module on the IMGT site. The complete DNA sequence of the V_L !1B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

```
Using neural networks (NN) and hidden Markov models (HMM) trained on eukaryotes


>Sequence

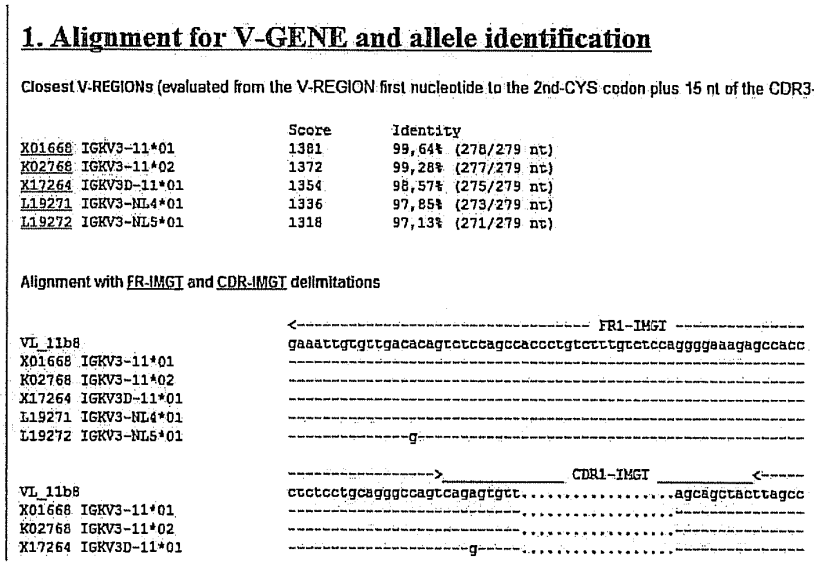

SignalP-NN result:
```




```
# data

>Sequence                 length = 70
# Measure  Position  Value  Cutoff  signal peptide?
  max. C    20        0.940   0.32   YES
  max. Y    20        0.883   0.33   YES
  max. S    13        0.988   0.87   YES
  mean S     1-19     0.930   0.48   YES
       D     1-19     0.907   0.43   YES
# Most likely cleavage site between pos. 19 and 20: VQC-EV
```

Figure 4
The V_H polypeptide sequence of antibody 7D8 was analyzed using the SignalP server. A screenshot of the resulting prediction of the signal peptide cleavage site is shown.



1. Alignment for V-GENE and allele identification

Closest V-REGIONs (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon plus 15 nt of the CDR3-IMGT)

	Score	Identity
X01668 IGKV3-11*01	1381	99,64% (278/279 nt)
K02768 IGKV3-11*02	1372	99,28% (277/279 nt)
X17264 IGKV3D-11*01	1354	98,57% (275/279 nt)
L19271 IGKV3-NL4*01	1336	97,85% (273/279 nt)
L19272 IGKV3-NL5*01	1318	97,13% (271/279 nt)

Alignment with FR-IMGT and CDR-IMGT delimitations

```
                          <---------------------------------- FR1-IMGT ----------------
VL_11b8                   gaaattgtgttgacacagtctccagccaccctgtctttgtctccaggggaaagagccacc
X01668 IGKV3-11*01        ---------------------------------------------------------------
K02768 IGKV3-11*02        ---------------------------------------------------------------
X17264 IGKV3D-11*01       ---------------------------------------------------------------
L19271 IGKV3-NL4*01       ---------------------------------------------------------------
L19272 IGKV3-NL5*01       ----------g----------------------------------------------------

                          ----------------->_____________ CDR1-IMGT _________<-----
VL_11b8                   ctctcctgcagggccagtcagagtgtt.................agcagctacttagcc
X01668 IGKV3-11*01        ---------------------------.................----------------
K02768 IGKV3-11*02        ---------------------------.................----------------
X17264 IGKV3D-11*01       ----------------------g----.................----------------
```


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(54) ANTI-TIM-3 ANTIBODY

(57) The present invention provides an anti-human TIM-3 antibody which binds to the amino acid sequence of the extracellular region of TIM-3 or its three-dimensional structure thereof and exhibits higher effector activity such as an antibody-dependent cellular cytotoxicity (ADCC activity) for diseases relating to a human TIM-3 expressing cell. The present invention provides a monoclonal antibody or antibody fragment thereof which binds to the amino acid sequence of the extracellular region of TIM-3 or its three-dimensional structure and exhibits ADCC activity; a hybridoma which produces the antibody; a DNA encoding the antibody; a vector comprising the DNA; a transformant which is obtainable by introducing the vector; a method for producing the antibody or the antibody fragment thereof which comprises using the hybridoma or the transformant; a therapeutic agent and a diagnostic agent comprising the antibody or the antibody fragment thereof as an active ingredient. In addition, the present invention provides an anti-human TIM-3 antibody having high ADCC activity by screening an anti-human TIM-3 antibody which competes with the monoclonal antibody or the antibody fragment thereof.

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Description

Technical Field

[0001] The present invention relates to a monoclonal antibody or an antibody fragment thereof, which binds to the extracellular region of T-cell immunoglobulin and mucin domain containing molecule-3 (hereinafter referred to as "TIM-3") and exhibits antibody-dependent cellular cytotoxicity (hereinafter referred to as "ADCC"); a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof.

Background Art

[0002] TIM-3 gene family consists of eight genes in mouse and three genes in human, and each of these genes are located at chromosome 11 and at chromosome 5q33 respectively (Non-patent Document 1). These gene regions are known to be related with autoimmune diseases and allergic diseases. TIM protein is a type I transmembrane protein having a structurally conserved immunoglobulin variable (IgV) domain and a mucin domain.

[0003] TIM protein was considered to be specifically expressed on T cells and directly regulate the T cell activity, but there are recent reports on expression of TIM-3 protein in antigen-presenting cells and on their functions (Non-patent Document 2). According to the crystal structure analysis, the TIM protein has a conserved protein structure and has a ligand binding site in the IgV domain.

[0004] TIM-3 was identified as a molecule specifically expressed on mouse Th1 cells but not on Th2 cells (Non-patent Document 3). The DNA sequence, the amino acid sequence and the three-dimensional structure of TIM-3 is available in the public data base such as the GenBank accession number NM_032782 and NM_134250. TIM-3 is also known as HAVCR2.

[0005] In humans, as similar to mice, TIM-3 is expressed on T-cells as well as phagocytic cells such as macrophages and dendritic cells. Binding of TIM-3 to a protein ligand (e.g., galectin-9) can inhibit the Th1 response via mechanism of apoptosis induction, and therefore lead to such as induction of peripheral tolerance.

[0006] The reduction in expression of human TIM-3 with siRNA or the inhibition of human TIM-3 by blocking-antibody increased the secretion of interferon γ (IFN-γ) from CD4 positive T-cells, supporting the inhibitory role of TIM-3 in human T cells. In phagocytes, TIM-3 also functions as a receptor for recognizing the apoptosis cells.

[0007] Analysis of clinical samples from autoimmune disease patients showed no expression of TIM-3 in CD4 positive cells. In particular, in T cell clones derived from the cerebrospinal fluid of patients with multiple sclerosis, the expression level of TIM-3 was lower and the secretion level of IFN-γ was higher than those of clones derived from normal healthy persons (Non-patent Document 4). There are reports on relation of TIM-3 with allergic diseases or asthma (Patent Documents 1 and 2).

[0008] According to the microarray analysis of hematopoietic stem cells from acute myeloid leukemia (hereinafter referred to as "AML") patients and normal hematopoietic stem cells, TIM-3 is expressed on AML stem cells and therefore the analysis suggested involvement of TIM-3 in hematological malignancy (Non-patent Document 5 and Patent Document 3).

[0009] Examples of the anti-TIM-3 monoclonal antibodies which were established up to now include anti-human TIM-3 rat monoclonal antibody (Clone 344823, manufactured by R&D Systems) and anti-human TIM-3 mouse monoclonal antibody (Clone F38-2E2, manufactured by R&D Systems).

Related Art

Pantent Literature

[0010]

Pantent Literature 1 WO96/27603
Pantent Literature 2 WO2003/063792
Pantent Literature 3 WO2009/091547

Non-Pantent Literature

[0011]

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Non-Pantent Literature 1 Hafler DA et al., JExp Med. 205: 2699-701 (2008)
Non-Pantent Literature 2 Anderson AC et al., Science 318: 1141-3 (2007)
Non-Pantent Literature 3 Monney L et al., Nature 415: 536-41 (2002)
Non-Pantent Literature 4 Koguchi K et al., J Exp Med. 203: 1413-8 (2006)
Non-Pantent Literature 5 Majeti R et al., Proc Natl Acad Sci USA 2009 Mar 3; 106 (9): 3396-401.

Disclosure of Invention

Problems to Be Solved by the Invention

[0012] However, there is no report on monoclonal antibody against human TIM-3 having ADCC activity. Therefore, the object of the present invention is to provide a monoclonal antibody or an antibody fragment which binds to the amino acid sequence or the three-dimensional structure of the extracellular region of TIM-3 and expresses ADCC activity. In addition, the object of the present invention is to provide an anti-human TIM-3 antibody having high ADCC activity by screening an anti-human TIM-3 antibody which competes with the monoclonal antibody or the antibody fragment thereof.
[0013] In addition, the present invention is to provide a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof as an active ingredient.

Means for Solving the Problems

[0014] The gist of the present invention relates to the followings.

(1) A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (i) to (iii):

(i) an antibody which comprises a heavy chain (hereinafter referred to as "H chain") of an antibody which comprises complementary determining regions (hereinafter referred to as "CDR") 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises a light chain (hereinafter referred to as "L chain") of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) an antibody which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) an antibody which comprises an H chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

(2) The monoclonal antibody or the antibody fragment thereof described in the above (1), wherein the monoclonal antibody binds to the same epitope to which one antibody selected from the above (i) to (iii) binds.
(3) A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (a) and (b):

(a) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

(4) The monoclonal antibody or the antibody fragment thereof described in the above (3), which binds to the same epitope to which one antibody selected from the above (a) or (b) binds.
(5) The monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (4), which is a recombinant antibody.
(6) The monoclonal antibody or the antibody fragment thereof described in the above (5), which is a recombinant antibody selected from a human chimeric antibody, a humanized antibody and a human antibody.
(7) A monoclonal antibody or an antibody fragment thereof, which is one selected from the following (i) to (iii):

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(i) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

(8) A monoclonal antibody and an antibody fragment thereof, which is one selected from the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

(9) A monoclonal antibody and an antibody fragment thereof which binds to the amino acid sequences at positions 67 to 105, the amino acid sequences at positions 67 to 96 or the amino acid sequences at positions 67 to 87 in the amino acid sequence of IgV domain of human TIM-3 represented by SEQ ID NO: 53.
(10) The antibody fragment described in any one of the above (1) to (9), wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv) and a peptide comprising CDR.
(11) A DNA which encodes the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(12) A recombinant vector which comprises the DNA described in the above (11).
(13) A transformant obtainable by introducing the recombinant vector described in the above (12) into a host cell.
(14) A process for producing the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10), which comprises culturing the transformant described in the above (13) in culture to form and accumulate the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10), and recovering the monoclonal antibody or the antibody fragment thereof from the culture.
(15) A method for immunologically detecting or measuring a human TIM-3 which comprises using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(16) A reagent for detecting or measuring a human TIM-3 which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(17) A diagnostic agent for a disease relating to a human TIM-3 positive cell which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(18) A method for diagnosing a disease relating to a human TIM-3 positive cell which comprises detect or measure a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(19) A method for diagnosing a disease relating to a human TIM-3 positive cell, which comprises ditecting or measuring a human TIM-3 using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(20) Use of the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10) for the manufacture of a diagnostic agent for a disease relating to a human TIM-3 positive cell.
(21) A therapeutic agent for a disease relating to a human TIM-3 positive cell, which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(22) A therapeutic method for a disease relating to a human TIM-3 positive cell, which comprises inducing cell death of a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(23) Use of the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10) for the manufacture of a therapeutic agent for a disease relating to a human TIM-3 positive cell.

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Effects of the Invention

[0015] The monoclonal antibody or the antibody fragment of the present invention specifically recognizes the amino acid sequence or the three-dimensional structure of the extracellular region of human TIM-3, and binds to the extracellular region. The amino acid sequence or the three-dimensional structure of the extracellular region of human TIM-3 which is recognized by the monoclonal antibody or the antibody fragment of the present invention is different from those recognized by known anti-TIM-3 monoclonal antibodies. Therefore, the monoclonal antibody or the antibody fragment of the present invention has higher ADCC activity. The monoclonal antibody or the antibody fragment of the present invention which specifically binds to the extracellular region of human TIM-3 and has higher ADCC activity is useful as a therapeutic agent and a diagnostic agent for a disease relating to a human TIM-3 positive cell.

[0016] The present invention may provide a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof.

Brief Description of the Drawings

[0017]

[Fig. 1] Fig. 1 is the amino acid sequences which represent HV0, HV3, HV4, HV5, HV6, HV7, HV8, HV10, and HV12 of the designed H chain variable regions of antibody 8213, respectively.

[Fig. 2] Fig. 2 is the amino acid sequences which represent LV0, LV2, LV4, LV5, LV6, LV7, and LV9 of the designed L chain variable regions of antibody 8213.

Embodiments for Carrying Out the Invention

[0018] The human TIM-3 of the present invention include a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782, and having a function of TIM-3; a polypeptide comprising an amino acid sequence having at least 60% homology, preferably at least 80% homology, more preferably at least 90% homology, and most preferably at least 95% homology, with the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782, and having a function of TIM-3; and the like.

[0019] The polypeptide comprising an amino acid sequence wherein one or more amino acid residue(s) is/are deleted, substituted and/or added in the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be obtained, for example, by introducing a site-directed mutation into DNA encoding the polypeptide comprising the amino acid sequence represented by SEQ ID NO: 53 by site-directed mutagenesis described in Molecular Cloning, A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory Press, 1989), Current Protocols in Molecular Biology (John Wiley & Sons, 1987-1997), Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci. USA, 79, 6409 (1982), Gene, 34, 315 (1985), Proc. Natl. Acad. Sci. USA, 82, 488 (1985), or the like.

[0020] The number of amino acid residues which are deleted, substituted or added is not particularly limited, and the number is preferably, 1 to dozens, such as 1 to 20, and more preferably 1 to several, such as 1 to 5.

[0021] As a gene which encodes human TIM-3, examples include the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782. Examples also include a gene comprising a nucleotide sequence in which at least one nucleotide is deleted, substituted or added in the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 and comprising a DNA encoding a polypeptide having a function of TIM-3; a gene comprising an nucleotide sequence having at least 60% homology, preferably at least 80% homology, and more preferably at least 95% homology, with the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 and comprising a DNA encoding a polypeptide having a function of TIM-3; a gene which hybridizes with the DNA consisting of the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 under stringent conditions and encodes a polypeptide having a function of TIM-3; and the like.

[0022] In the present invention, the DNA which hybridizes under stringent conditions refers to a DNA which is obtained by colony hybridization, plaque hybridization, Southern blot hybridization, DNA microarray analysis, or the like using a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 as a probe.

[0023] A specific example of such DNA is a DNA which can be identified by performing hybridization at 65°C in the presence of 0.7 to 1.0 mol/L sodium chloride using a filter or a slide glass with colony- or plaque-derived DNA, PCR products or oligo DNA encoding the DNA sequence immobilized thereon, and then washing the filter or a slide glass at 65°C with a 0.1 to 2-fold concentration SSC solution (1-fold concentration SSC solution: 150 mmol/L sodium chloride and 15 mmol/L sodium citrate). Hybridization can be carried out according to the methods described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular

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Biology, John Wiley & Sons (1987-1997); DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995); and the like.

[0024] Specifically, the DNA capable of hybridization includes DNA having at least 60% or more homology, preferably 80% or more homology, and more preferably 95% or more homology to the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782.

[0025] In the nucleotide sequence of the gene encoding a protein of a eukaryote, genetic polymorphism is often recognized. The TIM-3 gene used in the present invention also includes a gene in which small modification is generated in the nucleotide sequence by such polymorphism.

[0026] The number of the homology described in the present invention may be a number calculated by using a homology search program known by the skilled person, unless otherwise indicated. Regarding the nucleotide sequence, the number may be calculated by using a default parameter in BLAST [J. Mol. Biol., 215, 403 (1990)] or the like, and regarding the amino acid sequence, the number may be calculated by using a default parameter in BLAST2 [Nucleic Acids Res., 25, 3389 (1997); Genome Res., 7, 649 (1997); http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html] or the like.

[0027] As the default parameter, G (cost to open gap) is 5 for the nucleotide sequence and 11 for the amino acid sequence; -E (cost to extend gap) is 2 for the nucleotide sequence and 1 for the amino acid sequence; -q (penalty for nucleotide mismatch) is -3; -r (reward for nucleotide match) is 1; -e (expect value) is 10; -W (wordsize) is 11 residues for the nucleotide sequence and 3 residues for the amino acid sequence;.-y (Dropoff (X) for blast extensions in bits) is 20 for blastn and 7 for a program other than blastn; -X (X dropoff value for gapped alignment in bits) is 15; and Z (final X dropoff value for gapped alignment in bits) is 50 for blastn and 25 for a program other than blastn (http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html).

[0028] The polypeptide comprising a partial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be prepared according to a method known by the skilled person. For example, it can be prepared by deleting a part of DNA encoding the amino acid sequence represented by SEQ ID NO: 52 and culturing a transformant into which an expression vector containing the DNA is introduced.

[0029] Also, based on the thus prepared polypeptide or DNA, a polypeptide comprising an amino acid sequence in which one or more amino acid(s) is/are deleted, substituted or added in a partial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be prepared in the same manner as described above.

[0030] The polypeptide comprising a parcial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782; or the polypeptide comprising an amino acid sequence in which at least one amino acid is deleted, substituted or added in a parcial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can also be produced by a chemical synthesis method such as fluorenylmethoxycarbonyl (Fmoc) method or t-butyloxycarbonyl (tBoc) method.

[0031] The extracellular region of human TIM-3 of the present invention includes, for example, regions predicted by using the amino acid sequence of the polypeptide represented by SEQ ID NO: 53 with conventionally known transmembrane region prediction program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui submit.html), TMHMM ver. 2 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), ExPASy Proteomics Server (http://Ca.expasy.org/) or SMART (http://smart.embl-heidelberg.de/).

[0032] In the present invention, the amino acid sequence of extracellular region of human TIM-3 includes the amino acid sequence at positions 1 to 201 in the amino acid sequence represented by SEQ ID NO: 53, which is the region of the extracellular region predicted by SMART.

[0033] In the present invention, the three-dimensional structure of the extracellular region of human TIM-3 is not limited, so long as the extracellular region of human TIM-3 comprising the amino acid sequence represented by SEQ ID NO: 53 or GenBank accession number NM_032782 has the same structure as in the natural state. The three-dimensional structure of the extracellular region of human TIM-3 in the natural state is a natural type of the three-dimensional structure of human TIM-3 expressed on the surface of the cell membrane.

[0034] Regarding the function of human TIM-3, binding of TIM-3 with a protein ligand (e.g., galectin 9) can inhibit the Th1 response thorough mechanism such as apoptosis induction in Th1 cells, therefore leading to induction of peripheral tolerance. In phagocytes, human TIM-3 functions as a receptor for recognizing the apoptosis cells.

[0035] Binding of the antibody or antibody fragment thereof in the present invention to an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof can be confirmed by a conventionally known immunological immunological detection method using human TIM-3 expressing cells such as a radioimmuno assay with a solid phase sandwich method or enzyme-linked immunosorbent assay (ELISA), and preferably a fluorescent cell staining method in which the binding ability of a cell expressing a specified antigen and an antibody for the specific antigen is confirmed.

[0036] Specific examples include a fluorescent antibody staining method using an FMAT8100HTS System (manufactured by Applied Biosystems) [Cancer Immunol. Immunother, 36, 373 (1993)] or the like, a fluorescent cell staining

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method using flow cytometry, surface plasmon resonance using a Biacore System (manufactured by GE Healthcare) or the like, and the like.

[0037] Furthermore, a known immunological detection methods [Monoclonal Antibodies - Principles and practice, Third edition, Academic Press (1996), Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988), Monoclonal Antibody Experimental Manual, Kodan-sha Scientific (1987)] and the like can be combined to confirm the binding.

[0038] In the present invention, the cell expressing human TIM-3 may be any cell, so long as it expresses human TIM-3, and examples include a cell which is naturally present in the human body, a cell line established from the cell which is naturally present in the human body, a cell obtained by a recombinant technique and the like.

[0039] Example of the cell which is naturally present in the human body includes a cell expressing the TIM-3 in the body of a patient suffering from cancer, autoimmune disease or allergic disease, concretely, the cells are Th1 cells, macropharges, dendritic cells, and the like.

[0040] Example of a cell line established from the cell which is naturally present in the human body include a cell line expressing human TIM-3, among cell lines prepared by establishing the human TIM-3-expressing cells obtained from the above cancer patients, and examples include human acute myeloid leukemia cell line KG-1 (ATCC Accession No: CCL-246), human Burkitt's lymphoma cell line Daudi (ATCC Accession No: CCL-213) which are established from a human cell and the like.

[0041] Specific examples of the cell obtained by a recombinant technique may include a human TIM-3-expressing cell obtained by introducing an expression vector comprising a human TIM-3-encoding cDNA into an insect cell, an animal cell or the like, and others.

[0042] In addition, the present invention relates to a monoclonal antibody which recognizes an amino acid sequence or a three-dimensional structure of an extracellular region of human TIM-3 and exhibits ADCC activity.

[0043] The ADCC activity of the present invention is a reaction in which an antibody which is bound to human TIM-3 on the cell surface is bound to FcγRIIIa on the surface of mainly a natural killer cell (hereinafter referred to as NK cell) through Fc region and therefore a cytotoxic molecule such as molecules perforin, and granzyme released from an NK cell leads to cell lysis [Clark M, Chemical Immunology, 65, 88 (1997); Gorter A *et al.,* Immunol. Today, 20, 576 (1999)].

[0044] The antibody of the present invention may be any antibodies as long as it is an antibody or antibody fragment thereof which recognizes an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof and binds to the extracellular region; or an antibody or antibody fragment thereof which binds an extracellular region of human TIM-3 or a three-dimensional structure thereof and have ADCC activity.

[0045] Specific example of the antibody of the present invention may include an antibody which binds to the amino acid sequence selected from sequences preferably at positions 67 to 105, more preferably at positions 67 to 96, and most preferably at positions 67 to 87 among the amino acid sequence of extracellular region of human TIM-3 comprising of the sequence at positions 1 to 201 in the sequence represented by SEQ ID NO: 53, or the three-dimensional structure thereof.

[0046] The example of the antibody includes the monoclonal antibody and the antibody fragment thereof as described in the following (i) to (iii):

(i) a monoclonal antibody or an antibody fragment thereof which comprises a heavy chain (hereinafter referred to as "H chain") of an antibody which comprises complementary determining regions (hereinafter referred to as "CDR") 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises a light chain (hereinafter referred to as "L chain") of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively.

(ii) a monoclonal antibody or an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively.

(iii) a monoclonal antibody or an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

[0047] The example of the monoclonal antibody includes a monoclonal antibody and an antibody fragment thereof as described in the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,

(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the

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amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

[0048] In addition, the antibody of the present invention include a monoclonal antibody or an antibody fragment thereof which competes with the above monoclonal antibody in the binding of an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a monoclonal antibody or an antibody fragment thereof which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above monoclonal antibody binds.

[0049] In the present invention, an antibody which compete with a monoclonal antibody means an antibody which recognizes the same or a part of the same epitope (also referred to as antigen determinant) on the extracellular region of human TIM-3 as the monoclonal antibody of the present invention, and binds to the epitope. The antibody which binds to the same epitope as the monoclonal antibody of the present invention means an antibody which recognizes and binds to an amino acid sequence of human TIM-3 which the monoclonal antibody of the present invention recognizes.

[0050] The monoclonal antibody of the present invention includes an antibody produced by a hybridoma and a recombinant antibody produced by a transformant transformed with an expression vector containing a gene encoding an antibody.

[0051] The monoclonal antibody is an antibody secreted by a single clone antibody-producing cell, and recognizes only one epitope (also called antigen determinant) and has uniform amino acid sequence (primary structure).

[0052] Examples of the epitope include a single amino acid sequence, a three-dimensional structure comprising the amino acid sequence, a sugar chain-bound amino acid sequence, a three-dimensional structure comprising a sugar chain-bound amino acid sequence, and the like, recognized and bound by a monoclonal antibody.

[0053] The monoclonal antibody of the present invention binds preferably to the amino acid sequence at positions 22 to 131 of the IgV domain of human TIM-3 in the extracellular regions of human TIM-3 represented by SEQ ID NO: 53. Specifically, the amino acid sequence to which the monoclonal antibody of the present invention binds is preferably the amino acid sequence at positions 67 to 105, more preferably the amino acid sequence at positions 67 to 96, and most preferably the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0054] The epitope to which the monoclonal antibody of the present invention binds may preferably be included in the IgV domain of human TIM-3 represented by the amino acid sequence at positions 22 to 131 in the extracellular region of human TIM-3 represented by SEQ ID NO: 53. Specifically, the epitope to which the monoclonal antibody of the present invention binds is preferably included in the amino acid sequence at positions 67 to 105, more preferably included in the amino acid sequence at positions 67 to 96, and most preferably included in the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0055] The amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds may preferably include at least one amino acid selected from the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53 in the IgV domain of human TIM-3, and more preferably include at least one amino acid selected from the amino acids at position 67, position 74, position 76, position 78, position 79, position 81, position 83 and position 85.

[0056] The amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds may preferably include at least two or more continuous amino acids selected from positions 67 to 87 of the human TIM-3 IgV domain; and more preferably may include at least one amino acid selected from the amino acids at position 67, position 74, position 76, position 78, position 79, position 81, position 83 and position 85 and include at least two or more continuous amino acids selected from the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0057] Specifically, examples of the amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds include the amino acid sequence comprising the amino acids at positions 67 to 74, the amino acid sequence comprising the amino acids at positions 67 to 76, the amino acid sequence comprising the amino acids at positions 67 to 78, the amino acid sequence comprising the amino acids at positions 67 to 79, the amino acid sequence comprising the amino acids at positions 67 to 81, the amino acid sequence comprising the amino acids at positions 67 to 83, the amino acid sequence comprising the amino acids at positions 67 to 85,

the amino acid sequence comprising the amino acids at positions 74 to 76, the amino acid sequence comprising the amino acids at positions 74 to 78, the amino acid sequence comprising the amino acids at positions 74 to 79, the amino acid sequence comprising the amino acids at positions 74 to 81, the amino acid sequence comprising the amino acids at positions 74 to 83, the amino acid sequence comprising the amino acids at positions 74 to 85,

the amino acid sequence comprising the amino acids at positions 76 to 78, the amino acid sequence comprising the amino acids at positions 76 to 79, the amino acid sequence comprising the amino acids at positions 76 to 81, the amino acid sequence comprising the amino acids at positions 76 to 83, the amino acid sequence comprising the amino acids at positions 76 to 85, the amino acid sequence comprising the amino acids at positions 78 to 79, the amino acid sequence comprising the amino acids at positions 78 to 81, the amino acid sequence comprising the amino acids at positions 78

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to 83, the amino acid sequence comprising the amino acids at positions 78 to 85,
the amino acid sequence comprising the amino acids at positions 79 to 81, the amino acid sequence comprising the amino acids at positions 79 to 83, the amino acid sequence comprising the amino acids at positions 79 to 85,
the amino acid sequence comprising the amino acids at position 81 to 83, the amino acid sequence comprising the amino acids at positions 81 to 85,
the amino acid sequence comprising the amino acids at positions 83 to 85, and the like,
in the amino acid sequence represented by SEQ ID NO: 53.

[0058] The hybridoma can be prepared, for example, by preparing the above cell expressing TIM-3 as an antigen, inducing an antibody-producing cell having antigen specificity from an animal immunized with the antigen, and fusing the antibody-producing cell with a myeloma cell. The anti-TIM-3 antibody can be obtained by culturing the hybridoma or administering the hybridoma cell into an animal to cause ascites tumor in the animal and separating and purifying the culture or the ascites.

[0059] The animal immunized with an antigen may be any animal, so long as a hybridoma can be prepared, and mouse, rat, hamster, chicken, rabbit or the like is suitably used. Also, the cell having antibody-producing activity can be obtained from such an animal, and the antibody of the present invention includes an antibody produced by a hybridoma obtained by fusion of the cell after *in vitro* immunization with a myeloma cell.

[0060] In the present invention, the recombinant antibody includes an antibody produced by gene recombination, such as a human chimeric antibody, a human CDR grafted antibody, a human antibody and an antibody fragment thereof. Among the recombinant antibodies, one having character of a common monoclonal antibody, low immunogenecity and prolonged half-life in blood is preferable as a therapeutic agent. Examples of the recombinant antibody include an antibody which is prepared by modifying the above monoclonal antibody of the present invention using a recombinant technique.

[0061] The human chimeric antibody is an antibody comprising VH and VL of an antibody of a non-human animal, and a heavy chain constant region (hereinafter referred to as CH) and a light chain constant region (hereinafter referred to as CL) of a human antibody. Specifically, the human chimeric antibody of the present invention can be produced by obtaining cDNAs encoding VH and VL from a hybridoma which produces a monoclonal antibody which specifically recognizes human TIM-3 and binds to an amino acid sequence of extracellular region or a three-dimensional structure thereof, inserting each of them into an expression vector for animal cell comprising DNAs encoding CH and CL of human antibody to thereby construct a vector for expression of human chimeric antibody, and then introducing the vector into an animal cell to express the antibody.

[0062] As the CH of the human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg"), and those belonging to the hIgG class are preferred, and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. As the CL of the human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to κ class or λ class can be used.

[0063] Specific examples of the human chimeric antibody of the present invention includes a chimeric antibody which comprises VH of the antibody comprising the amino acid sequence represented by SEQ ID NO: 28 and comprises VL of the antibody comprising the amino acid sequence represented by SEQ ID NO: 30.

[0064] In addition, the chimeric antibody of the present invention include a chimeric antibody which competes with the above monoclonal antibody in the binding of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a chimeric antibody which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above chimeric antibody binds.

[0065] The human CDR grafted antibody is also called as a humanized antibody and is an antibody in which CDR of VH and VL of a non-human animal antibody were grafted into the appropriate site of VH and VL of a human antibody. The human CDR grafted antibody of the present invention can be produced by constructing cDNAs encoding an antibody V region in which the amino acid sequences of CDRs of VH and VL of an antibody derived from a non-human animal produced by a hybridoma which produces a monoclonal antibody which specifically recognizes three-dimensional structure of TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof are grafted into frameworks (hereinafter referred to as "FR") of VH and VL of a suitable human antibody, inserting each of them into an expression vector for animal cell comprising genes encoding CH and CL of a human antibody to thereby construct a vector for expression of humanized antibody, and introducing it into an animal cell to thereby express and produce the humanized antibody.

[0066] As the CH of the human CDR grafted antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg"), and those belonging to the hIgG class are preferred, and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. As the CL of the human CDR grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to κ class or λ class can be used.

[0067] Examples of the CDR grafted antibody of the present invention include a humanized antibody in which CDRs 1 to 3 of VH of the antibody comprises the amino acid sequences represented by SEQ ID NO: 21, 22, and 23, respectively,

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and CDRs 1 to 3 ofVL of the antibody comprises the amino acid sequences represented by SEQ ID NO: 24, 25, and 26, respectively.

[0068] Specific examples of the humanized antibody include a humanized antibody comprising at least one of the following (a) VH and (b) VL:

(a) VH of the antibody comprising the amino acid sequence represented by SEQ ID NO: 67, or an amino acid sequence in which at least one amino acid residue selected from Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 is substituted with other amino acid residue(s),

(b) VL of the antibody comprising the amino acid sequence represented by SEQ ID NO: 69, or an amino acid sequence in which at least one amino acid residue selected from Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 is substituted with other amino acid residue(s).

[0069] As the VH comprised in the humanized antibody, the following (1) to (8) are preferable:

(1) VH comprising an amino acid sequence in which Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(2) VH comprising an amino acid sequence in which Lys at position 12, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(3) VH comprising an amino acid sequence in which Val at position 20, Arg at position 38, Met at position 48, Val at position 68, Ile at position 70, Ala at position 72, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(4) VH comprising an amino acid sequence in which Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(5) VH comprising an amino acid sequence in which Lys at position 12, Arg at position 67, Val at position 68, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(6) VH comprising an amino acid sequence in which Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues,

(7) VH comprising an amino acid sequence in which Arg at position 38, Met at position 48, Arg at position 67 and Thr at position 74 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues; and

(8) VH comprising an amino acid sequence in which Arg at position 38, Met at position 48 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues.

[0070] The amino acid sequence of the above VH obtained by the amino acid modifications includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly or Val at position 113 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0071] Specific examples of the amino acid sequence of VH in which twelve modifications are introduced include an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0072] Examples of the amino acid sequence of VH in which eleven modifications are introduced include the following amino acid sequence (1) to (12):

(1) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at

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position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(3) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(5) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(6) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(7) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(8) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(9) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(10) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(11) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(12) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0073] Specific examples of the amino acid sequence of VH in which ten modifications are introduced include the following amino acid sequence (1) to (8):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced

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in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(8) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0074] Examples of the amino acid sequence of VH in which eight modifications are introduced include the following amino acid sequence (1) to (13):

(1) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at

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position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(11) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(12) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(13) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0075] Specific examples of the amino acid sequence of VH in which seven modifications are introduced include the following amino acid sequence (1) to (11):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(6) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(10) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0076] Specific Examples of the amino acid sequence of VH in which six modifications are introduced include the following amino acid sequence (1) to (16):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(12) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(13) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(14) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(15) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(16) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0077] Specific examples of the amino acid sequence of VH in which five modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0078] Specific examples of the amino acid sequence of VH in which four modifications are introduced include the following amino acid sequence (1) to (8):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0079] Specific examples of the amino acid sequence of VH in which three modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;

[0080] Specific examples of the amino acid sequence of VH in which two modifications are introduced include the following amino acid sequence (1) to (21):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Val at position 20 with Leu and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ala at position 40 with Arg are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(12) an amino acid sequence in which substitutions of Lys at position 12 with Val and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(13) an amino acid sequence in which substitutions of Val at position 20 with Leu and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(14) an amino acid sequence in which substitutions of Ala at position 40 with Arg and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(15) an amino acid sequence in which substitutions of Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(16) an amino acid sequence in which substitutions of Met at position 48 with Ile and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(17) an amino acid sequence in which substitutions of Met at position 48 with Ile and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(18) an amino acid sequence in which substitutions of Met at position 48 with Ile and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(19) an amino acid sequence in which substitutions of Met at position 48 with Ile and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(20) an amino acid sequence in which substitutions of Met at position 48 with Ile and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(21) an amino acid sequence in which substitutions of Met at position 48 with Ile and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0081] Specific examples of the amino acid sequence of VH in which one modification is introduced include the following amino acid sequence (1) to (12):

(1) an amino acid sequence in which substitution of Lys at position 12 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitution of Val at position 20 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(3) an amino acid sequence in which substitution of Arg at position 38 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitution of Ala at position 40 with Arg is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitution of Met at position 48 with Ile is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitution of Arg at position 67 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitution of Val at position 68 with Ala is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitution of Ile at position 70 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitution of Ala at position 72 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitution of Thr at position 74 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitution of Arg at position 98 with Gly is introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(12) an amino acid sequence in which substitution of Val at position 113 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0082] As the VL comprised in the humanized antibody, the following (1) to (6) are preferable:

(1) VL comprising an amino acid sequence in which Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(2) VL comprising an amino acid sequence in which Leu at position 11, Val at position 15, Tyr at position 36, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(3) VL comprising an amino acid sequence in which Val at position 15, Tyr at position 36, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(4) VL comprising an amino acid sequence in which Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(5) VL comprising an amino acid sequence in which Val at position 15, Tyr at position 36, Leu at position 46 and Phe at position 71 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(6) VL comprising an amino acid sequence in which Tyr at position 36 and Pro at position 44 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.

[0083] The amino acid sequence of the above VL obtained by the amino acid modifications includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr, and Thr at position 85 with Asp is introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0084] Examples of the amino acid sequence of VL in which nine modifications are introduced include an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr, and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0085] Specific examples of the amino acid sequence of VL in which eight modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

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(2) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(3) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(7) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(8) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and

(9) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0086] Specific example of the amino acid sequence of VL in which seven modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(3) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(7) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(8) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at

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position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(9) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0087] Specific example of the amino acid sequence of VL in which six modifications are introduced include the following amino acid sequence (1) to (6):

(1) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0088] Specific example of the amino acid sequence of VL in which five modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0089] Specific example of the amino acid sequence of VL in which four modifications are introduced include the following amino acid sequence (1) to (10):

(1) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ

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ID NO: 69;
(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(9) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(10) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0090] Specific example of the amino acid sequence ofVL in which three modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Ala at position 13 with Val, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0091] Specific example of the amino acid sequence of VL in which two modifications are introduced include the following amino acid sequence (1) to (15):

(1) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Leu at position 11 with Met and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Ala at position 13 with Val and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;

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(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Ala at position 43 with Ser are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(9) an amino acid sequence in which substitutions of Leu at position 11 with Met and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(10) an amino acid sequence in which substitutions of Ala at position 13 with Val and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(11) an amino acid sequence in which substitutions of Val at position 15 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(12) an amino acid sequence in which substitutions of Ala at position 43 with Ser and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(13) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(14) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(15) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0092] Specific example of the amino acid sequence of VH in which one modification is introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitution of Leu at position 11 with Met is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitution of Ala at position 13 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitution of Val at position 15 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitution of Tyr at position 36 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitution of Ala at position 43 with Ser is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitution of Pro at position 44 with Phe is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitution of Leu at position 46 with Gly is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitution of Phe at position 71 with Tyr is introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(9) an amino acid sequence in which substitution of Thr at position 85 with Asp is introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0093] In addition, examples of the humanized antibody of the present invention include a humanized antibody in which VH of the antibody comprises the amino acid sequence represented by SEQ ID NO: 67 and/or VL of the antibody comprises the amino acid sequence represented by SEQ ID: 69, a humanized antibody in which VH of the antibody comprises the amino acid sequence represented by SEQ ID NO: 67 and/or VL of the antibody comprises any one of the amino acid sequences shown in Fig. 2, or a humanized antibody in which VH of the antibody comprises any one of the amino acid sequences shown in Fig. 1 and/or VL of the antibody comprises the amino acid sequence represented by SEQ ID: 69.

[0094] Furthermore, examples of the humanized antibody of the present invention include a humanized antibody which competes with the monoclonal antibody of the present invention in the binding of an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a humanized antibody which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above humanized antibody binds.

[0095] A human antibody is originally an antibody naturally existing in the human body, and it also includes an antibody obtained from a human antibody phage library or a human antibody-producing transgenic animal, which is prepared

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based on the recent advanced techniques in genetic engineering, cell engineering and developmental engineering.

[0096] The antibody existing in the human body can be prepared, for example, by isolating a human peripheral blood lymphocyte, immortalizing it by infecting with EB virus or the like, and then cloning it to thereby obtain lymphocytes capable of producing the antibody, culturing the lymphocytes thus obtained, and purifying the antibody from the supernatant of the culture.

[0097] The human antibody phage library is a library in which antibody fragments such as Fab and scFv are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the index. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

[0098] A human antibody-producing transgenic animal is an animal in which a human antibody gene is integrated into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a gene encoding a human antibody into a mouse ES cell, grafting the ES cell into an early embryo of mouse and then developing it. A human antibody is prepared from the human antibody-producing transgenic non-human animal by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in non-human mammals, culturing the obtained hybridoma and forming and accumulating the human antibody in the supernatant of the culture.

[0099] In the amino acid sequence constituting the above antibody or antibody fragment thereof, an antibody or antibody fragment thereof in which one or more amino acids are deleted, substituted, inserted or added, having activity similar to the above antibody or antibody fragment thereof is also included in the antibody or antibody fragment of the present invention.

[0100] The number of amino acids which are deleted, substituted, inserted and/or added is one or more, and is not specifically limited, but it is within the range where deletion, substitution or addition is possible by known methods such as the site-directed mutagenesis described in *Molecular Cloning,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* John Wiley & Sons (1987-1997); *Nucleic Acids Research,* 10, 6487 (1982), *Proc. Natl. Acad. Sci. USA,* 79, 6409 (1982); *Gene,* 34, 315 (1985), *Nucleic Acids Research,* 13, 4431 (1985); *Proc. Natl. Acad. Sci. USA,* 82, 488 (1985) or the like. For example, the number is preferably 1 to dozens, more preferably 1 to 20, furthermore preferably 1 to 10, and most preferably 1 to 5.

[0101] The expression "one or more amino acid residue(s) is/are deleted, substituted, inserted and/or added" in the amino acid sequence of the above antibody means the followings. That is, it means there is deletion, substitution, insertion or addition of one or plural amino acids at optional positions in the same sequence and in one or plural amino acid sequences. Also, the deletion, substitution, insertion or addition may occur at the same time and the amino acid which is substituted, inserted or added may be either a natural type or a non-natural type.

[0102] The natural type amino acid includes L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-cysteine and the like.

[0103] Preferable examples of mutually substitutable amino acids are shown below. The amino acids in the same group are mutually substitutable.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine

Group B: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid

Group C: asparagine, glutamine

Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid

Group E: proline, 3-hydroxyproline, 4-hydroxyproline

Group F: serine, threonine, homoserine

Group G: phenylalanine, tyrosine

[0104] The antibody fragment of the present invention includes Fab, $F(ab')_2$, Fab', a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv), a peptide comprising CDR and the like.

[0105] An Fab is an antibody fragment among fragments obtained by treating an IgG antibody molecule with a protease, papain (cleaved at an amino acid residue at position 224 of the H chain), having a molecular weight of about 50,000 and having antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain are bound together through a disulfide bond.

[0106] The Fab of the present invention can be produced by treating the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with papain. Also, the Fab can be produced by inserting DNA encoding Fab of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to express the Fab.

[0107] An $F(ab')_2$ is an antibody fragment having a molecular weight of about 100,000 and having antigen binding

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activity and comprising two Fab regions which are bound in the hinge position obtained by digesting the lower part of two disulfide bonds in the hinge region of IgG with an enzyme, pepsin.

[0108] The $F(ab')_2$ of the present invention can be produced by treating the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with pepsin. Also, the $F(ab')_2$ can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

[0109] An Fab' is an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, which is obtained by cleaving a disulfide bond at the hinge region of the above $F(ab')_2$. The Fab' of the present invention can be produced by treating the $F(ab')_2$ of the present invention which specifically recognizes TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with a reducing agent, such as dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to express the Fab'.

[0110] An scFv is a VH-P-VL or VL-P-VH polypeptide in which one chain VH and one chain VL are linked using an appropriate peptide linker (hereinafter referred to as "P") and is an antibody fragment having antigen binding activity.

[0111] The scFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv.

[0112] A diabody is an antibody fragment wherein scFv is dimerized, is an antibody fragment having divalent antigen binding activity. In the divalent antigen binding activity, two antigens may be the same or different.

[0113] The diabody of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding scFv so that the length of the amino acid sequence of the peptide linker is 8 or less residues, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the diabody.

[0114] A dsFv is obtained by binding polypeptides in which one amino acid residue of each of VH and VL is substituted with a cysteine residue via a disulfide bond between the cysteine residues. The amino acid residue to be substituted with a cysteine residue can be selected based on a three-dimensional structure estimation of the antibody in accordance with a known methods *[*Protein Engineering, 7, 697 (1994)].

[0115] The dsFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding dsFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the dsFv.

[0116] A peptide comprising CDR is constituted by including one or more regions of CDRs of VH or VL. Peptide comprising plural CDRs can be bound directly or via an appropriate peptide linker.

[0117] The peptide comprising CDR of the present invention can be produced by constructing DNA encoding CDRs of VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the peptide. The peptide comprising CDR can also be produced by a chemical synthesis method such as Fmoc method or tBoc method.

[0118] The monoclonal antibody of the present invention includes an antibody conjugate in which the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof is chemically or genetically bound to a radioisotope, an agent having a low molecular weight, an agent having a high molecular weight, a protein, a therapeutic antibody or the like.

[0119] The antibody conjugate of the present invention can be produced by chemically conjugating a radioisotope, an agent having a low molecular weight, an agent having a high molecular weight, an adjuvant, a protein, a therapeutic antibody or the like to the N-terminal side or C-terminal side of an H chain or an L chain, adequate substituent or sidechain, sugarchain, and the like of the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof *[*Kotai Kogaku Nyumon, published by Chijin Shokan (1994)].

[0120] Also, the antibody conjugate can be genetically produced by linking a DNA encoding the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid

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sequence of the extracellular region or three-dimensional structure thereof to other DNA encoding a protein or a therapeutic antibody to be conjugated, inserting the DNA into a vector for expression, and introducing the expression vector into an appropriate host cell.

[0121] The radioisotope includes ^{131}I, ^{125}I, ^{90}Y, ^{64}Cu, ^{199}Tc, ^{77}Lu, ^{211}At and the like. The radioisotope can directly be conjugated with the antibody by Chloramine-T method or the like. Also, a substance chelating the radioisotope can be conjugated with the antibody. The chelating agent includes 1-isothiocyanatobenzyl-3-methyldiethylenetriaminepentaacetic acid (MX-DTPA) and the like.

[0122] The agent having a low molecular weight includes an anti-tumor agent such as an alkylating agent, a nitrosourea agent, a metabolism antagonist, an antibiotic substance, an alkaloid derived from a plant, a topoisomerase inhibitor, an agent for hormonotherapy, a hormone antagonist, an aromatase inhibitor, a P glycoprotein inhibitor, a platinum complex derivative, an M-phase inhibitor and a kinase inhibitor *[*Rinsho Syuyo-gaku (Clinical Oncology), Gan to Kagakuryoho-Sha (1996)], a steroid agent such as hydrocortisone and prednisone, a nonsteroidal agent such as aspirin and indomethacin, immunomodulatory agent such as aurothiomalate, penicillamine, immuno-suppressing agent such as cyclophosphamide and azathioprine, antiinflammatory agent such as anti-histamine agent, for example, chlorpheniramine maleate and clemastine *[*Ensho to Kouensho-Ryoho (Inflammation and Anti-inflammation Therapy), Ishiyaku Shuppann (1982)] and the like.

[0123] Examples of the antitumor agent include amifostine (Ethyol), cisplatin, dacarbazine (DTIC), dactinomycin, mecloretamin (nitrogen mustard), streptozocin, cyclophosphamide, iphosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), epirubicin, gemcitabine (Gemsal), daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, fluorouracil, vinblastine, vincristine, bleomycin, daunomycin, peplomycin, estramustine, paclitaxel (Taxol), docetaxel (Taxotea), aldesleukin, asparaginase, busulfan, carboplatin, oxaliplatin, nedaplatin, cladribine, camptothecin, 10-hydroxy-7-ethylcamptothecin (SN38), floxuridine, fludarabine, hydroxyurea, iphosphamide, idarubicin, mesna, irinotecan (CPT-11), nogitecan, mitoxantrone, topotecan, leuprolide, megestrol, melfalan, mercaptopurine, hydroxycarbamide, plicamycin, mitotane, pegasparagase, pentostatin, pipobroman, streptozocin, tamoxifen, goserelin, leuprorelin, flutamide, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil, hydrocortisone, prednisolone, methylprednisolone, vindesine, nimustine, semustine, capecitabine, Tomudex, azacytidine, UFT, oxaliplatin, gefitinib (Iressa), imatinib (STI 571), elrotinib, FMS-like tyrosine kinase 3 (Flt3) inhibitor, vascular endothelial growth facotr receptor (VEGFR) inhibitor, fibroblast growth factor receptor (FGFR) inhibitor, Epidermal Growth Factor Receptor (EGFR) inhibitor such as Iressa and Tarceva, radicicol, 17-allylamino-17-demethoxygeldanamycin, rapamycin, amsacrine, all-trans-retinoic acid, thalidomide, anastrozole, fadrozole, letrozole, exemestane, gold thiomalate, D-penicillamine, bucillamine, azathioprine, mizoribine, cyclosporine, rapamycin, hydrocortisone, bexarotene (Targretin), tamoxifen, dexamethasone, progestin substances, estrogen substances, anastrozole (Arimidex), Leuplin, aspirin, indomethacin, celecoxib, azathioprine, penicillamine, gold thiomalate, chlorpheniramine maleate, chlorpheniramine, clemastine, tretinoin, bexarotene, arsenic, voltezomib, allopurinol, calicheamicin, ibritumomab tiuxetan, Targretin, ozogamine, clarithromycin, leucovorin, ifosfamide, ketoconazole, aminoglutethimide, suramin, methotrexate, maytansinoid and derivatives thereof.

[0124] The method for conjugating the agent having low molecular weight with the antibody includes a method in which the agent and an amino group of the antibody are conjugated through glutaraldehyde, a method in which an amino group of the agent and a carboxyl group of the antibody are conjugated through water-soluble carbodiimide, and the like.

[0125] The agent having a high molecular weight includes polyethylene glycol (hereinafter referred to as "PEG"), albumin, dextran, polyoxyethylene, styrene-maleic acid copolymer, polyvinylpyrrolidone, pyran copolymer, hydroxypropylmethacrylamide, and the like. By binding these compounds having a high molecular weight to an antibody or antibody fragment, the following effects are expected: (1) improvement of stability against various chemical, physical or biological factors, (2) remarkable prolongation of half life in blood, (3) disappearance of immunogenicity or suppression of antibody production, and the like *[*Bioconjugate Drug, Hirokawa Shoten (1993)]. For example, the method for binding PEG to an antibody includes a method in which an antibody is allowed to react with a PEG-modifying reagent [Bioconjugate Drug, Hirokawa Shoten (1993)]. The PEG-modifying reagent includes a modifying agent of ε-amino group of lysine (Japanese Published Unexamined Patent Application No. 178926/86), a modifying agent of a carboxyl group of aspartic acid and glutamic acid (Japanese Published Unexamined Patent Application No. 23587/81), a modifying agent of a guanidino group of arginine (Japanese Published Unexamined Patent Application No. 117920/90) and the like.

[0126] The immunostimulator may be any natural products known as immunoadjuvants. Examples of an agent enhancing immunogen include $\beta(1\rightarrow3)$glucan (lentinan, schizophyllan), α-galactosylceramide and the like.

[0127] The protein includes a cytokine or a growth factor which activates a immunocompetent cell, such as NK cell, macrophage or neutrophil, a toxic protein, and the like.

[0128] Examples of the cytokine or the growth factor include interferon (hereinafter referred to as "IFN")-α, IFN-β, IFN-γ, interleukin (hereinafter referred to as "IL")-2, IL-12, IL-15, IL-18, IL-21, IL-23, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) and the like.

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[0129] The toxic protein includes ricin, diphtheria toxin, ONTAK and the like, and also includes a toxic protein wherein mutation is introduced into a protein in order to control the toxicity.

[0130] The therapeutic antibody includes an antibody against an antigen in which apoptosis is induced by binding of the antibody, an antibody against an antigen participating in formation of pathologic state of tumor, an antibody against an antigen regulating immunological function and an antibody against an antigen relating to angiogenesis in the pathologic part.

[0131] The antigen in which apoptosis is induced by binding of the antibody includes cluster of differentiation (hereinafter "CD") 19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85, CD86 (B7.2), human leukocyte antigen (HLA)-Class II, epidermal growth factor receptor (EGFR) and the like.

[0132] The antigen participating in formation of pathologic state of tumor or the antigen regulating immunological function includes CD40, CD40 ligand, B7 family molecule (CD80, CD86, CD274, B7-DC, B7-H2, B7-H3, B7-H4), ligand of B7 family molecule (CD28, CTLA-4, ICOS, PD-1, BTLA), OX-40, OX-40 ligand, CD137, tumor necrosis factor (TNF) receptor family molecule (DR4, DR5, TNFR1, TNFR2), TNF-related apoptosis-inducing ligand receptor (TRAIL) family molecule, receptor family of TRAIL family molecule (TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4), receptor activator of nuclear factor kappa B (RANK), RANK ligand, CD25, folic acid receptor 4, cytokine [IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, transforming growth factor (TGF) β, TNFα, etc.], receptors of these cytokines, chemokine (SLC, ELC, I-309, TARC, MDC, CTACK, *etc.*) and receptors of these chemokines.

[0133] The antigen for the antibody which inhibits angiogenesis in the pathologic part includes vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), EGF, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), erythropoietin (EPO), TGFβ, IL-8, Ephilin, SDF-1, receptors thereof and the like.

[0134] A fusion antibody with a protein or therapeutic antibody can be produced by linking a cDNA encoding a monoclonal antibody or antibody fragment to a cDNA encoding the protein, constructing a DNA encoding the fusion antibody, inserting the DNA into an expression vector for prokaryote or eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the fusion antibody.

[0135] In the case where the above antibody conjugate is used for the detection method, method for quantitative determination, detection reagent, reagent for quantitative determination or diagnostic agent in the present invention, examples of the agent to which the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof is bound includes a label used in routine immunological detecting or measuring method.

[0136] The label includes enzymes such as alkaline phosphatase, peroxidase and luciferase, luminescent materials such as acridinium ester and lophine, fluorescent materials such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (RITC), and the like.

[0137] Furthermore, the present invention includes an agent for treating a disease relating to a TIM-3 positive cell, comprising the monoclonal antibody of the present invention or the antibody fragment thereof as an active ingredient.

[0138] The disease relating to the TIM-3 positive cell is not limited, so long as it is a disease relating to a cell expressing TIM-3, such as cancer, autoimmune disease, and allergic disease.

[0139] The cancer includes blood cancer, breast cancer, uterine cancer, colorectal cancer, esophageal cancer, gastric cancer, ovarian cancer, lung cancer, renal cancer, rectal cancer, thyroid cancer, uterine cervix cancer, small intestinal cancer, prostate cancer and pancreatic cancer. Preferable examples of the cancer include blood cancer, esophageal cancer, gastric cancer, colorectal cancer, liver cancer and prostate cancer.

[0140] Examples of the blood cancer include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplasticsyndromes (MDS), multiple myeloma, cutaneous T cell lymphoma (CTCL), peripheral T-cell lymphoma (PTCL), anaplastic large cell lymphoma (ALCL), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), other lymphoid leukemia, NK cell lymphoma, Hodgkin lymphoma, non-Hodgkin's lymphoma such as Burkitt's lymphoma, and the like.

[0141] Specific examples of the autoimmune diseases include rheumatoid arthritis, psoriasis, Crohn's disease, Ankylosing spondylitis, multiple sclerosis, type I diabetes, hepatitis, myocarditis, Sjogren's syndrome, autoimmune hemolytic anemia after transplant rejection, blisters pemphigoid, Graves disease, Hashimoto's thyroiditis, systemic lupus erythematosus (SLE), myasthenia gravis, pemphigus, pernicious anemia, and the like.

[0142] Examples of the allergic diseases include acute or chronic reactive airway disease, bronchial asthma, atopic dermatitis, allergic rhinitis, urticaria, PIE syndrome, food allergies, hay fever, allergic nose, bronchial asthma, atopic dermatitis, anaphylactic shock and the like.

[0143] The therapeutic agent of the present invention comprises the above monoclonal antibody or the antibody fragment of the present invention as an active ingredient.

[0144] The therapeutic agent comprising the monoclonal antibody of the present invention or antibody fragment thereof, or conjugate thereof may comprise only the antibody or antibody fragment thereof, or conjugate thereof as an active ingredient. It is generally preferred that the therapeutic agent is prepared as a pharmaceutical preparation produced by

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an appropriate method well known in the technical field of pharmaceutics, and by mixing it with one or more pharmaceutically acceptable carriers.

[0145] It is preferred to administer the therapeutic agent by the route that is most effective for the treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration and intravenous administration is preferred.

[0146] The pharmaceutical preparation includes sprays, capsules, tablets, powders, granules, syrups, emulsions, suppositorys, injections, ointments, tapes and the like.

[0147] Although the dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 μg/kg to 10 mg/kg per day and per adult.

[0148] Further, the present invention relates to a method for immunologically detecting or measuring TIM-3, a reagent for immunologically detecting or measuring TIM-3, a method for immunologically detecting or measuring a cell expressing TIM-3, and a diagnostic agent for diagnosing a disease relating to a TIM-3 positive cell, comprising the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof as an active ingredient.

[0149] In the present invention, the method for detecting or measuring the amount of TIM-3 may be any known method. For example, it includes an immunological detecting or measuring method.

[0150] The immunological detecting or measuring method is a method in which an antibody amount or an antigen amount is detected or determined using a labeled antigen or antibody. Examples of the immunological detecting or measuring method include radioactive substance-labeled immunoantibody method (RIA), enzyme immunoassay (EIA or ELISA), fluorescent immunoassay (FIA), luminescent immunoassay, Western blotting method, physicochemical method and the like.

[0151] The above disease relating to a TIM-3 positive cell can be diagnosed by detecting or measuring a cell expressing TIM-3 by using the monoclonal antibody or antibody fragment of the present invention.

[0152] For the detection of the cell expressing the polypeptide, known immunological detection methods can be used, and an immunoprecipitation method, a fluorescent cell staining method, an immune tissue staining method and the like are preferably used. Also, a fluorescent antibody staining method using FMAT 8100 HTS system (Applied Biosystem) and the like can be used.

[0153] In the present invention, the living body sample to be used for detecting or measuring TIM-3 is not particularly limited, so long as it has a possibility of containing a cell expressing TIM-3, such as tissue cells, blood, blood plasma, serum, pancreatic fluid, urine, fecal matter, tissue fluid or culture fluid.

[0154] The diagnostic agent containing the monoclonal antibody of the present invention or antibody fragment thereof, or conjugate thereof may further contain a reagent for carrying out an antigen-antibody reaction or a reagent for detection of the reaction depending on the desired diagnostic method. The reagent for carrying out the antigen-antibody reaction includes a buffer, a salt, and the like. The reagent for detection includes a reagent generally used for the immunological detecting or measuring method, such as labeled secondary antibody which recognizes the monoclonal antibody, antibody fragment thereof or conjugates thereof and substrate corresponding to the labeling and the like.

[0155] A process for producing the antibody of the present invention, a method for treating the disease and a method for diagnosing the disease are specifically described below.

1. Preparation method of monoclonal antibody

(1) Preparation of antigen

[0156] TIM-3 or a cell expressing TIM-3 as an antigen can be obtained by introducing an expression vector comprising cDNA encoding a full length of TIM-3 or a partial length thereof is introduced into *Escherichia coli,* yeast, an insect cell, an animal cell or the like. In addition, TIM-3 can be purified and obatained from various human tumor cell lines, human tissue and the like which express a large amount of TIM-3. The tumor cell line and the tissue can be directly allowed to use as antigens. Furthermore, a synthetic peptide having a partial sequence of the TIM-3 can be prepared by a chemical synthesis method such as Fmoc method or tBoc method and used as an antigen.

[0157] TIM-3 used in the present invention can be produced, for example, by expressing a DNA encoding TIM-3 in a host cell using a method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) or the like according to the following method.

[0158] Firstly, a recombinant vector is prepared by inserting a full length cDNA comprising the region encoding TIM-3 into downstream of a promoter of an appropriate expression vector. At this time, if necessary, a DNA fragment having an appropriate length containing a region encoding the polypeptide prepared based on the full length cDNA may be used instead of the above full length cDNA. Next, a transformant producing polypeptide can be obtained by introducing

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the recombinant vector into a host cell suitable for the expression vector.

[0159] The expression vector may be any one, so long as it can replicate autonomously in the host cell to be used or it can be integrated into a chromosome comprising an appropriate promoter at such a position that the DNA encoding the polypeptide can be transcribed.

[0160] The host cell may be any one, so long as it can express the objective gene. Examples include a microorganism which belongs to the genera *Escherichia,* such as *Escherichia coli,* yeast, an insect cell, an animal cell and the like.

[0161] When a prokaryote such as *Escherichia coli* is used as the host cell, it is preferred that the recombinant vector used in the present invention is autonomously replicable in the prokaryote and comprising a promoter, a ribosome binding sequence, the DNA encoding TIM-3 and a transcription termination sequence. The recombinant vector is not necessary to have a transcription termination sequence, but a transcription termination sequence is preferably set just below the structural gene. The recombinant vector may further comprise a gene regulating the promoter.

[0162] Also, the above recombinant vector is preferably a plasmid in which the space between Shine-Dalgarno sequence, which is the ribosome binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides).

[0163] Furthermore, the nucleotide sequence of the DNA encoding TIM-3 can be substituted with another base so as to be a suitable codon for expressing in a host cell, thereby improve the productivity of the objective TIM-3.

[0164] Any expression vector can be used, so long as it can function in the host cell to be used. Examples of the expression vector includes pBTrp2, pBTac1, pBTac2 (all manufactured by Roche Diagnostics), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 *[*Agric. Biol. Chem., 53, 277 (1989)], pGEL1 *[*Proc. Natl. Acad Sci. USA, 82, 4306 (1985)], pBluescript II SK(-) (manufactured by Stratagene), pTrs30 [prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)], pTrs32 [prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)], pGHA2 [prepared from *Escherichia coli* IGHA2 (FERM BP-400), Japanese Published Unexamined Patent Application No. 221091/85], pGKA2 [prepared from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/85], pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (manufactured by Pharmacia), pET system (manufactured by Novagen), pME18SFL3 and the like.

[0165] Any promoter can be used, so long as it can function in the host cell to be used. Examples include promoters derived from *Escherichia coli,* phage and the like, such as trp promoter (Ptrp), lac promoter, PL promoter, PR promoter and T7 promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two Ptrp are tandemly linked, tac promoter, lacT7 promoter and letI promoter, can be used.

[0166] Examples of host cell include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* DH5α and the like.

[0167] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA into the host cell, and examples include a method using a calcium ion described in Proc. Natl. Acad. Sci. USA, 69, 2110 (1972), Gene, 17, 107 (1982) and Molecular & General Genetics, 168, 111 (1979) and the like.

[0168] When an animal cell is used as the host cell, any expression vector can be used, so long as it can function in the animal cell. Examples include pcDNAI, pcDM8 (manufactured by Funakoshi), pAGE107 [Japanese Published Unexamined Patent Application No. 22979/91; Cytotechnology, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [Nature, 329, 840,(1987)], pcDNAI/Amp (manufactured by Invitrogen), pcDNA3.1 (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J. Biochemistry, 101, 1307 (1987)], pAGE210, pME18SFL3, pKANTEX93 (WO 97/10354) and the like.

[0169] Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of immediate early (IE) gene of cytomegalovirus (CMV), SV40 early promoter, a promoter of retrovirus, a metallothionein promoter, a heat shock promoter, SRα promoter, Molony murine leukemia virus promoter or enhancer, and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

[0170] The host cell includes human Namalwa leukemia cell, monkey COS cell, Chinese hamster ovary (CHO) cell [Journal of Experimental Medicine, 108, 945 (1958); Proc.Natl.Acad.Sci. USA, 60, 1275 (1968*);*Genetics, 55, 513(1968); Chromosoma, 41, 129 (1973); Methods in Cell Science, 18, 115 (1996); Radiation Research, 148, 260 (1997); Proc.Natl.Acad.Sci. USA, 77, 4216 (1980); Proc.Natl.Acad.Sci. USA, 60, 1275 (1968); Cell, 6, 121 (1975); Molecular Cell genetics, Appendix I, II (pp.883-900)], CHO/DG44, CHO-K1 (ATCC accession NO: CCL-61), DUkXB11 (ATCC accession NO:CCL-9096), Pro-5 (ATCC accession NO: CCL-1781), CHO-S (Life Technologies, Cat#11619), Pro-3, rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also called as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14, Syrian hamster cell BHK or HBT5637 (Japanese Published Unexamined Patent Application No. 299/88) and the like.

[0171] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA

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into an animal cell, and examples include electroporation [Cytotechnology, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the like.

[0172] TIM-3 can be produced by culturing the transformant derived from a microorganism, an animal cell or the like having a recombinant vector comprising the DNA encoding TIM-3 in a medium to form and accumulate TIM-3 in the culture, and recovering it from the culture. The method for culturing the transformant in the medium is carried out according to the usual method used in culturing of hosts.

[0173] When TIM-3 is expressed in a cell derived from eukaryote, TIM-3 to which sugars or sugar chains bind can be obtained.

[0174] When a microorganism transformed with a recombinant vector containing an inducible promoter is cultured, an inducer can be added to the medium, if necessary. For example, isopropyl-β-D-thiogalactopyranoside or the like can be added to the medium when a microorganism transformed with a recombinant vector using *lac* promoter is cultured; or indoleacrylic acid or the like can be added thereto when a microorganism transformed with a recombinant vector using trp promoter is cultured.

[0175] When a transformant obtained using an animal cell as the host cell is cultured, the medium includes generally used RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], Dulbecco's modified MEM medium [Virology, 8, 396 (1959)] and 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)], Iscove's modified Dulbecco's medium (IMDM), the media to which fetal calf serum, *etc.* is added, and the like. Culture is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO_2. If necessary, an antibiotic such as kanamycin or penicillin can be added to the medium during the culturing.

[0176] Regarding the expression method of the gene encoding TIM-3, in addition to direct expression, secretory production, fusion protein expression and the like can be carried out according to the method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

[0177] The process for producing TIM-3 includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, a method of producing on a host cell outer membrane, and the like. The appropriate method can be selected by changing the host cell used and the structure of the TIM-3 produced.

[0178] When the TIM-3 is produced in a host cell or on a host cell membrane outer envelope, TIM-3 can be positively secreted extracellularly in accordance with the method of Paulson et al. [J. Biol. Chem., 264, 17619 (1989)], the method of Lowe et al. [Proc. Natl. Acad. Sci. USA, 86, 8227 (1989), Genes Develop., 4, 1288 (1990)], the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and WO 94/23021, and the like.

[0179] Also, the production amount of TIM-3 can be increased in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 utilizing a gene amplification system using a dihydrofolate reductase gene.

[0180] The resulting TIM-3 can be isolated and purified, for example, as follows.

[0181] When TIM-3 is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonicator, French press, Manton Gaulin homogenizer, dynomill or the like to obtain a cell-free extract.

[0182] The cell-free extract is centrifuged to obtain a supernatant, and a purified preparation can be obtained by subjecting the supernatant to a general protein isolation and purification techniques such as solvent extraction; salting out with ammonium sulfate etc.; desalting; precipitation with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical); cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia); hydrophobic chromatography using a resin such as butyl-Sepharose or phenyl-Sepharose; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing; and the like which may be used alone or in combination.

[0183] When TIM-3 is expressed intracellularly by forming an inclusion body, the cells are recovered, disrupted and centrifuged in the same manner, and the inclusion body of TIM-3 are recovered as a precipitation fraction. The recovered inclusion body of the TIM-3 protein is solubilized with a protein denaturing agent. The protein is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified preparation of polypeptide is obtained by the same isolation purification method as above.

[0184] When TIM-3 or the derivative such as a glycosylated product is secreted extracellularly, TIM-3 or the derivative such as a glycosylated product can be recovered from the culture supernatant. That is, the culture is treated by a method such as centrifugation in the same manner as above to obtain a soluble fraction, a purified preparation of TIM-3 can be obtained from the soluble fraction by the same isolation purification method as above.

[0185] Also, TIM-3 used in the present invention can be produced by a chemical synthesis method, such as Fmoc method or tBoc method. Also, it can be chemically synthesized using a peptide synthesizer manufactured by Advanced ChemTech, Perkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corpo-

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ration, or the like.

(2) Immunization of animal and preparation of antibody-producing cell for fusion

[0186] A mouse, rat or hamster and the like which is 3 to 20-weeks-old is immunized with the antigen prepared in the above (1), and antibody-producing cells within the spleen, lymph node or peripheral blood of the animal are collected. Also, when the increase of a sufficient titer in the above animal is not recognized due to low immunogenecity, a TIM-3 knockout mouse may by used as an animal to be immunized.

[0187] The immunization is carried out by administering the antigen to the animal through subcutaneous, intravenous or intraperitoneal injection together with an appropriate adjuvant (for example, complete Freund's adjuvant, combination of aluminum hydroxide gel with pertussis vaccine, or the like). When the antigen is a partial peptide, a conjugate is produced with a carrier protein such as BSA (bovine serum albumin), KLH (keyhole limpet hemocyanin) or the like, which is used as the antigen.

[0188] The administration of the antigen is carried out 5 to 10 times every one week or every two weeks after the first administration. On the 3rd to 7th day after each administration, a blood sample is collected from the fundus of the eye, the reactivity of the serum with the antigen is tested, for example, by enzyme immunoassay [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like. An animal showing a sufficient antibody titer in their sera against the antigen used for the immunization is used as the source of antibody-producing cells for fusion.

[0189] Three to seven days after final administration of the antigen, tissue containing the antibody-producing cells such as the spleen is excised from the immunized animal to collect the antibody-producing cells. When the spleen cells are used, the spleen is cut out and loosened, followed by centrifugation. Then, antibody-producing cells for fusion are obtained by removing erythrocytes.

(3) Preparation of myeloma cell

[0190] An established cell line obtained from mouse is used as myeloma cells. Examples include 8-azaguanine-resistant mouse (derived from BALB/c) myeloma cell line P3-X63Ag8-U1 (P3-U1) [Current Topics in Microbiology and Immunology, 18, 1 (1978)], P3-NS1/1-Ag41 (NS-1) [European J. Immunology, 6, 511 (1976)], SP2/0-Ag14 (SP-2) [Nature, 276, 269 (1978)], P3-X63-Ag8653 (653) [J. Immunology, 123, 1548 (1979)], P3-X63-Ag8 (X63) [Nature, 256, 495 (1975)] and the like.

[0191] The myeloma cells are subcultured in a normal medium [a medium in which glutamine, 2-mercaptoethanol, gentamicin, FBS and 8-azaguanine are added to RPMI-1640 medium] and they are subcultured in the normal medium 3 or 4 days before cell fusion to ensure the cell number of 2×10^7 or more on the day for fusion.

(4) Cell fusion and preparation of hybridoma for producing monoclonal antibody

[0192] The antibody-producing cells for fusion obtained by the above (2) and myeloma cells obtained by the above (3) were sufficiently washed with a minimum essential medium (MEM) or PBS (1.83 g of disodium hydrogen phosphate, 0.21 g of potassium dihydrogen phosphate, 7.65 g of sodium chloride, 1 liter of distilled water, pH 7.2) and mixed to give a ratio of the antibody-producing cells: the myeloma cells = 5 to 10: 1, followed by centrifugation. Then, the supernatant is discarded.

[0193] The precipitated cell group is sufficiently loosened. After loosening the precipitated cell, the mixture of polyethylene glycol-1000 (PEG-1000), MEM and dimethylsulfoxide is added to the cell under stirring at 37°C. In addition, 1 to 2 mL of MEM medium is added several times every one or two minutes, and MEM is added to give a total amount of 50 mL. After centrifugation, the supernatant is discarded. After the cells are gently loosened, the cells are gently suspended in HAT medium [a normal medium containing hypoxanthine, thymidine and aminopterin]. The suspension is cultured in a 5% CO_2 incubator for 7 to 14 days at 37°C.

[0194] After the culturing, a portion of the culture supernatant is sampled and a hybridoma which is reactive to an antigen containing TIM-3 and is not reactive to an antigen not containing TIM-3 is selected by binding assay as described below. Then, cloning is carried out twice by a limiting dilution method [Firstly, HT medium (HAT medium from which aminopterin is removed) is used, and secondly, the normal medium is used], and a hybridoma which stably shows a high antibody titer is selected as the monoclonal antibody-producing hybridoma.

(5) Preparation of purified monoclonal antibody

[0195] The hybridoma cells producing a monoclonal antibody obtained by the above (4) are administered by intraperitoneal injection into 8- to 10-week-old mice or nude mice treated with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane (pristane) is intraperitoneally administered, followed by feeding for 2 weeks). The hybridoma develops ascites

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tumor in 10 to 21 days. The ascitic fluid is collected from the mice, centrifuged to remove solids, subjected to salting out with 40 to 50% ammonium sulfate and then precipitated by caprylic acid, passed through a DEAE-Sepharose column, a protein A column or a gel filtration column to collect an IgG or IgM fraction as a purified monoclonal antibody.

[0196] Furthermore, a monoclonal antibody-producing hybridoma obtained by the above (4) is cultured in RPMI1640 medium containing 10% FBS or the like and the supernatant is removed by centrifugation. The precipitated cells are suspended in Hybridoma-SFM medium and cultured for 3 to 7 days. The purified monoclonal antibody can be obtained by centrifusing the obtained cell suspension, followed by purification from the resulting supernatant with Protein A column or Protein G column to collect the IgG fractions. Additionally, Hybridoma-SFM medium can contain 5% DIGO GF21.

[0197] The subclass of the antibody can be determined using a subclass typing kit by enzyme immunoassay. The amount of the protein can be determined by the Lowry method or from the absorbance at 280 nm.

(6) Selection of monoclonal antibody

[0198] Selection of monoclonal antibody is carried out by the following binding assay using an enzyme immunoassay method and inhibition assay of intracellular uptake of amino acids.

(6-a) Binding assay

[0199] As the antigen, a gene-introduced cell obtained by introducing an expression vector containing a cDNA encoding TIM-3 obtained in (1) into *Escherichia coli,* yeast, an insect cell, an animal cell or the like, a recombinant protein, or a purified polypeptide or partial peptide obtained from a human tissue is used. When the antigen is a partial peptide, a conjugate is prepared with a carrier protein such as BSA or KLH is prepared and is used.

[0200] After making these antigens into a solid layer by dispensing in a 96-well plate, a substance to be tested such as serum, a culture supernatant of a hybridoma or a purified monoclonal antibody is dispensed therein as the primary antibody and allowed to react. After thoroughly washing with PBS, PBS-Tween and the like, an anti-immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent material, a radiation compound or the like is dispensed therein as the secondary antibody and allowed to react. After thoroughly washing with PBS-Tween, the reaction appropriate to the label of the secondary antibody is carried out to select a monoclonal antibody which specifically reacts with the antigen.

[0201] In addition, a monoclonal antibody which competes with the anti-TIM-3 monoclonal antibody of the present invention in the binding of an extracellular region of human TIM-3 can be obtained by adding a subject antibody to the above binding assay system to allow to react. Namely, a monoclonal antibody which competes with the obtained monoclonal antibody in the binding of an amino acid sequence of the extracellular region of human TIM-3 or a three-dimensional structure thereof can be obtained by screening a subject antibody which inhibits the binding of the monoclonal antibody upon adding to the antibody.

[0202] Furthermore, an antibody which binds to the same epitope to which the monoclonal antibody of the present invention which binds to an amino acid sequence of the extracellular region of TIM-3 or three-dimensional structure thereof can be obtained by identifying an epitope of the antibody obtained by the above binding assay system, preparing a partial synthetic peptide of the epitope or a synthetic peptide which mimics the three-dimensional structure of the epitope and immunizing with the peptide.

(6-b) Kinetic analysis with Biacore

[0203] The kinetics between an antigen and a test substance is measured using Biacore T100 and then the obtained results are analyzed using analysis software accompanied with the apparatus. After anti-mouse IgG antibody is immobilized onto to a CM 5 sensor chip by an amine coupling method, a test substance such as culture supernatant of a hybridoma, a purified monoclonal antibody is allowed to flow and bind at an appropriate amount, and an antigen at plural known concentrations is further allowed to flow. And then, the binding and dissociation are measured.

[0204] Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using the 1:1 binding model to obtain necessary parameters. Otherwise, after human TIM-3 is immobilized onto the sensor chip by an amino coupling method, a purified monoclonal antibody is allowed to flow at plural known concentrations followed by measuring the binding and dissociation. Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using bivalent analyte model to obtain necessary parameters.

2. Preparation of recombinant antibody

[0205] As production examples of recombinant antibodies, processes for producing a human chimeric antibody and a human CDR grafted antibody are shown below.

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(1) Construction of vector for expression of recombinant antibody

[0206] A vector for expression of recombinant antibody is an expression vector for animal cell into which DNAs encoding CH and CL of a human antibody have been inserted, and is constructed by cloning each of DNAs encoding CH and CL of a human antibody into an expression vector for animal cell.

[0207] The C region of a human antibody may be used CH and CL of any human antibody. Examples include CH belonging to γ1 subclass, CL belonging to κ class, and the like. As the DNAs encoding CH and CL of a human antibody, the cDNA may be generally used and a chromosomal DNA comprising an exon and an intron can be also used.

[0208] As the expression vector for animal cell, any expression vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [Cytotechnol., 3, 133 (1990)], pAGE103 [J. Biochem., 101, 1307 (1987)], pHSG274 [Gene, 27, 223 (1984)], pKCR [Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)], pSG1bd2-4 [Cytotechnol., 4, 173 (1990)], pSE1UK1Sed1-3 [Cytotechnol., 13, 79 (1993)] and the like.

[0209] Examples of a promoter and enhancer used for an expression vector for animal cell include an SV40 early promoter [J. Biochem., 101, 1307 (1987)], a Moloney mouse leukemia virus LTR [Biochem. Biophys. Res. Commun., 149, 960 (1987)], an immunoglobulin H chain promoter [Cell, 41, 479 (1985)] and enhancer [Cell, 33, 717 (1983)] and the like.

[0210] The vector for expression of recombinant antibody may be either of a type in which a gene encoding an antibody H chain and a gene encoding an antibody L chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a vector for expression of recombinant antibody, easiness of introduction into animal cells, and balance between the expression amounts of antibody H and L chains in animal cells, a tandem type of the vector for expression of recombinant antibody is more preferred [J. Immunol. Methods, 167, 271 (1994)]. Examples of the tandem type of the vector for expression of recombinant antibody include pKANTEX93 (WO 97/10354), pEE18 [Hybridoma, 17, 559 (1998)], and the like.

(2) Obtaining of cDNA encoding V region of antibody derived from non-human animal and analysis of amino acid sequence

[0211] Obtaining of cDNAs encoding VH and VL of a non-human antibody and analysis of amino acid sequence are carried out as follows.

[0212] mRNA is extracted from hybridoma cells producing a non-human antibody to synthesize cDNA. The synthesized cDNA is cloned into a vector such as a phage or a plasmid, to prepare a cDNA library.

[0213] Each of a recombinant phage or recombinant plasmid containing cDNA encoding VH or VL is isolated from the library using DNA encoding a part of the C region or V region of a mouse antibody as the probe. The full length of the nucleotide sequences of VH and VL of a mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and the full length of the amino acid sequences of VH and VL are deduced from the nucleotide sequences, respectively.

[0214] Examples of the non-human animal for preparing a hybridoma cell which produces a non-human antibody include mouse, rat, hamster, rabbit or the like. Any animals can be used so long as a hybridoma cell can be produced therefrom.

[0215] Examples of the method for preparing total RNA from a hybridoma cell include a guanidine thiocyanate-cesium trifluoroacetate method [Methods in Enzymol., 154, 3 (1987)], the use of a kit such as RNA easy kit (manufactured by Qiagen) and the like.

[0216] Examples of the method for preparing mRNA from total RNA include an oligo (dT) immobilized cellulose column method [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)], a method using a kit such as Oligo-dT30 <Super> mRNA Purification Kit (manufactured by Takara Bio) and the like. Additionary, examples of the method for preparing mRNA include method using a kit Fast Track mRNA Isolation kit (manufactured by Invitrogen) or QuickPrep mRNA Purification Kit (manufactured by Pharmacia) and the like.

[0217] Examples of the method for synthesizing cDNA and preparing a cDNA library include known methods [*Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* Supplement 1, John Wiley & Sons (1987-1997)]; a method using a kit such as Super Script Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Invitrogen), ZAP-cDNA Synthesis Kit (manufactured by Stratagene), *etc.;* and the like.

[0218] The vector into which the synthesized cDNA using mRNA extracted from a hybridoma cell as the template is inserted for preparing a cDNA library may be any vector, so long as the cDNA can be inserted. Examples include ZAP ExPress [*Strategies, 5,* 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research,* 17, 9494 (1989)], λZAPII (manufactured by Stratagene), λgt10 and λgt11 [*DNA Cloning: A Practical Approach,* I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell and pT7T3-18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.,* 3, 280 (1983)], pUC18 [*Gene,* 33, 103 (1985)], and the like.

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[0219] Any *Escherichia coli* for introducing the cDNA library constructed by a phage or plasmid vector may be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF' [*Strategies,* 5, 81 (1992)], C600 [*Genetics,* 39, 440 (1954)], Y1088 and Y1090 [*Science, 222:* 778 (1983)], NM522 [*J. Mol. Biol.,* 166, 1 (1983)], K802 [*J. Mol. Biol.,* 16, 118 (1966)], JM105 [*Gene,* 38, 275 (1985)], and the like.

[0220] A colony hybridization or plaque hybridization method using an isotope- or fluorescence-labeled probe may be used for selecting cDNA clones encoding VH or VL of a non-human antibody or the like from the cDNA library [*Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989)].

[0221] Also, the cDNAs encoding VH and VL can be prepared through polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* Supplement 1, John Wiley & Sons (1987-1997)) by preparing primers and using cDNA prepared from mRNA or a cDNA library as the template.

[0222] The nucleotide sequence of the cDNA can be determined by digesting the cDNA selected with appropriate restriction enzymes and the like, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), carrying out the reaction by a usually used nucleotide sequence analyzing method. For example, a nucleotide sequence analyzing method is carried out by using an automatic nucleotide sequence analyzer such as ABI PRISM3700 (manufactured by PE Biosystems) and A.L.F. DNA sequencer (manufactured by Pharmacia) after a reaction such as the dideoxy method *[*Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)].

[0223] Whether the obtained cDNAs encode the full amino acid sequences of VH and VL of the antibody containing a secretory signal sequence can be confirmed by estimating the full length of the amino acid sequences of VH and VL from the determined nucleotide sequence and comparing them with the full length of the amino acid sequences of VH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)].

[0224] With regard to the full amino acid sequences of VH and VL of the antibody containing a secretory signal sequence, the length of the secretory signal sequence and N-terminal amino acid sequence can be estimated by comparing them with the full length of the amino acid sequences ofVH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)], and furthermore the subgroup to which they belong can be determined. In addition, the amino acid sequence of each CDR of VH and VL can be determined by comparing them with the full length of the amino acid sequences of VH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)].

[0225] Moreover, the novelty of the full length of the amino acid sequence of VH and VL can be examined by carrying out a homology search with sequences in any database, for example, SWISS-PROT, PIR-Protein or the like using the obtained full length of the amino acid sequences of VH and VL, for example, according to the BLAST method [J. Mol. Biol., 215, 403 (1990)] or the like.

(3) Construction of vector for expression of human chimeric antibody

[0226] cDNA encoding each of VH and VL of antibody of non-human animal is cloned in the upstream of genes encoding CH or CL of human antibody of vector for expression of recombinant antibody mentioned in the above (1) to thereby construct a vector for expression of human chimeric antibody.

[0227] In order to ligate cDNA comprising a nucleotide sequence of 3'-terminal of VH or VL of antibody of non-human animal and a nucleotide sequence of 5'-terminal of CH or CL of human antibody, each cDNA encoding VH or VL of a non-human animal antibody is prepared so as to encodes appropriate amino acids and have an appropriate recognition sequence of a restriction enzyme at a linkage portion.

[0228] The prepared cDNAs encoding VH and VL of antibody are respectively cloned so that each of them is expressed in an appropriate form in the upstream of gene encoding CH or CL of the human antibody of the vector for expression of the human CDR-grafted antibody mentioned in the above (1) to construct a vector for expression of human chimeric antibody.

[0229] In addition, cDNA encoding VH or VL of a non-human animal antibody is amplified by PCR using a synthetic DNA having a recognition sequence of an appropriate restriction enzyme at both ends and each of them is cloned to the vector for expression of recombinant antibody obtained in the above (1).

(4) Construction of cDNA encoding V region of human CDR-grafted antibody

[0230] cDNAs encoding VH or VL of a human CDR-grafted antibody can be obtained as follows.

[0231] Amino acid sequences of framework region (hereinafter referred to as "FR") in VH or VL of a human antibody to which amino acid sequences of CDRs in VH or VL of an antibody derived from a non-human animal antibody are transplanted are respectively selected. Any amino acid sequences of FR of a human antibody can be used, so long as they are derived from human.

[0232] Examples include amino acid sequences of FRs of human antibodies registered in database such as Protein

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Data Bank or the like, and amino acid sequences common to subgroups of FRs of human antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the like. In order to inhibit the decrease in the binding activity of the antibody, amino acid sequences having high homology (at least 60% or more) with the amino acid sequence of FR in VH or VL of the original antibody is selected.

[0233] Then, amino acid sequences of CDRs of the original antibody are grafted to the selected amino acid sequence of FR in VH or VL of the human antibody, respectively, to design each amino acid sequence of VH or VL of a humanized antibody. The designed amino acid sequences are converted to DNA sequences by considering the frequency of codon usage found in nucleotide sequences of genes of antibodies [Sequence of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the DNA sequence encoding the amino acid sequence of VH or VL of a humanized antibody is designed.

[0234] Based on the designed nucleotide sequences, several synthetic DNAs having a length of about 100 nucleotides are synthesized, and PCR is carried out using them. In this case, it is preferred that 6 synthetic DNAs per each of the H chain and the L chain are designed in view of the reaction efficiency of PCR and the lengths of DNAs which can be synthesized.

[0235] Furthermore, the cDNA encoding VH or VL of a human CDR-grafted antibody can be easily cloned into the vector for expression of human CDR-grafted antibody constructed in (1) by introducing the recognition sequence of an appropriate restriction enzyme to the 5' terminal of the synthetic DNAs existing on the both ends.

[0236] Otherwise, it can be carried out using a synthetic DNA as one DNA encoding each of the full-length H chain and the full-length L chain based on the designed DNA sequence.

[0237] After the PCR, an amplified product is cloned into a plasmid such as pBluescript SK (-) (manufactured by Stratagene) or the like, and the nucleotide sequence is determined according to a method similar to the method described in (2) to obtain a plasmid having a DNA sequence encoding the amino acid sequence of VH or VL of a desired humanized antibody.

(5) Modification of amino acid sequence of V region of human CDR-grafted antibody

[0238] It is known that when a human CDR-grafted antibody is produced by simply grafting only CDRs in VH and VL of an antibody derived from a non-human animal into FRs of VH and VL of a human antibody, its antigen binding activity is lower than that of the original antibody derived from a non-human animal [BIO/TECHNOLOGY, 9, 266 (1991)].

[0239] In human CDR-grafted antibodies, among the amino acid sequences of FRs in VH and VL of a human antibody, an amino acid residue which directly relates to binding to an antigen, an amino acid residue which interacts with an amino acid residue in CDR, and an amino acid residue which maintains the three-dimensional structure of an antibody and indirectly relates to binding to an antigen are identified and modified to an amino acid residue which is found in the original non-humanized antibody to thereby increase the antigen binding activity which has been decreased.

[0240] In order to identify the amino acid residues relating to the antigen binding activity in FR, constructing the three-dimensional structure of an antibody and analyzing can be done by X-ray crystallography [J. Mol. Biol., 112, 535 (1977)], computer-modeling [Protein Engineering, 7, 1501 (1994)] or the like. In addition, modified human CDR-grafted antibody having sufficient binding activity against antigen can be obtained by various attempts, such as producing several modified antibodies of each antibody and examining their binding activities.

[0241] The modification of the amino acid sequence of FR in VH and VL of a human antibody can be accomplished using various synthetic DNA for modification according to PCR as described in (4). With regard to the amplified product obtained by the PCR, the nucleotide sequence is determined according to the method as described in (2) so that whether the objective modification has been carried out is confirmed.

(6) Construction of vector for expression of human CDR-grafted antibody

[0242] A vector for expression of human CDR-grafted antibody can be constructed by cloning each cDNA encoding VH or VL of a constructed recombinant antibody into upstream of each gene encoding CH or CL of the human antibody in the vector for expression of recombinant antibody as described in (1).

[0243] For example, when recognizing sequences of an appropriate restriction enzymes are introduced to the 5'-terminal of synthetic DNAs positioned at both ends among synthetic DNAs used in the construction of VH or VL of the human CDR-grafted antibody in (4) and (5), cloning can be carried out so that they are expressed in an appropriate form in the upstream of each gene encoding CH or CL of the human antibody in the vector for expression of a human CDR-grafted antibody as described in (1).

(7) Transient expression of recombinant antibody

[0244] In order to efficiently evaluate the antigen binding activity of various human CDR-grafted antibodies produced,

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the recombinant antibodies can be expressed transiently using the vector for expression of antibody as described in (3) and (6) or the modified expression vector thereof.

[0245] Any cell can be used as a host cell, so long as the host cell can express a recombinant antibody. For example, COS-7 cell (ATCC CRL1651) is used in view of its high expression amount *[*Methods in Nucleic Acids Res., CRC Press, 283 (1991)].

[0246] Examples of the method for introducing the expression vector into COS-7 cell include a DEAE-dextran method *[Methods in Nucleic Acids Res.,* CRC Press, 283 (1991)], a lipofection method *[Proc. Natl. Acad. Sci. USA,* 84, 7413 (1987)], and the like.

[0247] After introduction of the expression vector, the expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be determined by the enzyme immunoassay [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988), Monoclonal Antibody Experiment Manual, Kodansha Scientific (1987)] and the like.

(8) Obtaining transformant which stably expresses recombinant antibody and preparation of recombinant antibody

[0248] A transformant which stably expresses a recombinant antibody can be obtained by introducing the vector for expression of recombinant antibody described in (3) and (6) into an appropriate host cell.

[0249] Examples of the method for introducing the expression vector into a host cell include electroporation [Japanese Published Unexamined Patent Application No. 257891/90, Cytotechnology, 3, 133 (1990)] and the like.

[0250] As the host cell into which a vector for expression of a recombinant antbody is introduced, any cell can be used, so long as it is a host cell which can produce the recombinant antibody. Examples include CHO-K1 (ATCC CCL-61), DUkXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S (Life Technologies, Cat # 11619), rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also referred to as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14 (ATCC No. CRL1581), mouse P3X63-Ag8653 cell (ATCC No. CRL1580), CHO cell in which a dihydrofolate reductase gene (hereinafter referred to as "dhfr") is defective [Proc. Natl. Acad. Sci. U.S.A., 77, 4216 (1980)], lection resistance-acquired Lec 13 [Somatic Cell and Molecular genetics, 12, 55 (1986)], CHO cell in which α1,6-fucosyltransaferse gene is defected (WO 2005/35586, WO 02/31140), rat YB2/3HL.P2.G11.16Ag.20 cell (ATCC No. CRL1662), and the like.

[0251] In addition, host cells in which activity of a protein such as an enzyme relating to synthesis of an intracellular sugar nucleotide, GDP-fucose, a protein such as an enzyme relating to the modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α-bond in a complex type N-glycoside-linked sugar chain, or a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose, to the Golgi body is decreased or deleted, preferably CHO cell in which α1,6-fucosyltransferase gene is defected as described in W02005/35586, WO02/31140 or the like, can also be used.

[0252] After introduction of the expression vector, transformants which express a recombinant antibody stably are selected by culturing in a medium for animal cell culture containing an agent such as G418 sulfate (hereinafter referred to as "G418") or the like (Japanese Published Unexamined Patent Application No. 257891/90).

[0253] Examples of the medium for animal cell culture include RPMI1640 medium (manufactured by Invitrogen), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL301 medium (manufactured by JRH), IMDM medium (manufactured by Invitrogen), Hybridoma-SFM medium (manufactured by Invitrogen), media obtained by adding various additives such as FBS to these media, and the like.

[0254] The recombinant antibody can be produced and accumulated in a culture supernatant by culturing the obtained transformants in a medium. The expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be measured by ELISA or the like. Also, in the transformant, the expression amount of the recombinant antibody can be increased by using DHFR amplification system or the like according to the method disclosed in Japanese Published Unexamined Patent Application No. 257891/90.

[0255] The recombinant antibody can be purified from the culture supernatant of the transformant by using a protein A column [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)]. In addition, the recombinant antibody can be purified by a combination of the methods for purification such as gel filtration, ionexchange chromatography, ultrafiltration and the like.

[0256] The molecular weight of the H chain or the L chain of the purified recombinant antibody or the antibody molecule as a whole is determined by polyacrylamide gel electrophoresis (hereinafter referred to as "SDS-PAGE") *[*Nature, 227, 680 (1970)], Western blotting [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], and the like.

3. Activity evaluation of the monoclonal antibody or antibody fragment

[0257] The activity of the purified monoclonal antibody or antibody fragment of the present invention can be evaluated in the following manner.

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[0258] The binding activity to TIM-3-expressing cell is evaluated by the binding assay described in the above 1-(6a) and a surface plasmon resonance method using such as the Biacore system described in the above (6b). Furthermore, it can be measured by fluorescent antibody technique [Cancer Immunol. Immunother, 36, 373 (1993)] and the like.

[0259] Complement-Dependent Cytotoxicity (hereinafter, referred to as "CDC activity") or ADCC activity against an antigen positive cell line is evaluated by a known method [Cancer Immunol. Immunother., 36, 373 (1993)].

4. Method of controlling effector activity of antibody

[0260] As a method for controlling an effector activity of the monoclonal antibody of the present invention, a method for controlling an amount of fucose (hereinafter, referred to also as "core fucose") which is bound in α-1,6 linkage to N-acetylglucosamine (G1cNAc) present in a reducing end of a complex type N-linked sugar chain which is bound to asparagine (Asn) at position 297 of an Fc region of an antibody (W02005/035586, W02002/31140, and WO00/61739), a method for controlling an effector activity of a monoclonal antibody by modifying amino acid group(s) of an Fc region of the antibody, and the like are known. The effector activity of the anti-TIM-3 monoclonal antibody of the present invention can be controlled by using any of the methods.

[0261] The "effector activity" means an antibody-dependent activity which is induced via an Fc region of an antibody. As the effector activity, ADCC activity, CDC activity, an antibody-dependent phagocytosis (ADP activity) by phagocyte such as macrophages or dendritic cells, and the like are known.

[0262] By controlling a content of core fucose of a complex type N-linked sugar chain of Fc of an antibody, an effector activity of the antibody can be increased or decreased. According to a method for lowering a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of the antibody, an antibody to which fucose is not bound can be obtained by the expression of an antibody using a CHO cell which is deficient in a gene encoding α1,6-fucosyltransferase. The antibody to which fucose is not bound has a high ADCC activity.

[0263] On the other hand, according to a method for increasing a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of an antibody, an antibody to which fucose is bound can be obtained by the expression of an antibody using a host cell into which a gene encoding α1,6-fucosyltransferase is introduced. The antibody to which fucose is bound has a lower ADCC activity than the antibody to which fucose is not bound.

[0264] Further, by modifying amino acid residue(s) in an Fc region of an antibody, the ADCC activity or CDC activity can be increased or decreased. For example, the binding activity to an antibody can be increased by using the amino acid sequence of the Fc region described in US2007/0148165. Further, the ADCC activity or CDC activity can be increased or decreased by modifying the amino acid as described in US Patent Nos. 6,737,056, or 7,297,775 or 7,317,091.

[0265] Furthermore, an antibody in which the effector activity is controlled can be obtained by combining the above method within one antibody.

5. Method for treating disease using the anti-TIM-3 monoclonal antibody or antibody fragment of the present invention

[0266] The monoclonal antibody or an antibody fragment thereof of the present invention can be used for treating a disease relating to a TIM-3 positive cell.

[0267] The therapeutic agent comprising the antibody or antibody fragment of the present invention or derivatives thereof may be only the antibody or antibody fragment or derivatives thereof as an active ingredient, and is preferably supplied as a pharmaceutical preparation produced by an appropriate method well known in the technical field of pharmaceutics, by mixing it with one or more pharmaceutically acceptable carriers.

[0268] Examples of a route of administration include oral administration and non-oral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration. Examples of the dosage form includes sprays, capsules, tablets, powder, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

[0269] The pharmaceutical preparation suitable for oral administration includes emulsions, syrups, capsules, tablets, powders, granules and the like.

[0270] Liquid preparations such as emulsions and syrups can be produced using, as additives, water; sugars such as sucrose, sorbitol and fructose; glycols such as polyethylene glycol and propylene glycol; oils such as sesame oil, olive oil and soybean oil; antiseptics such as p-hydroxybenzoic acid esters; flavors such as strawberry flavor and peppermint; and the like.

[0271] Capsules, tablets, powders, granules and the like can be produced using, as additives, excipients such as lactose, glucose, sucrose and mannitol; disintegrating agents such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose and gelatin; surfactants such as fatty acid ester; plasticizers such as glycerin; and the like.

[0272] The pharmaceutical preparation suitable for parenteral administration includes injections, suppositories, sprays and the like.

[0273] Injections can be prepared using a carrier such as a salt solution, a glucose solution or a mixture of both thereof.

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[0274] Suppositories can be prepared using a carrier such as cacao butter, hydrogenated fat or carboxylic acid.

[0275] Sprays can be prepared using the antibody or antibody fragment as such or using it together with a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the compound by dispersing it as fine particles. The carrier includes lactose, glycerol and the like. It is possible to produce pharmaceutical preparations such as aerosols and dry powders.

[0276] In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

6. Method for diagnosing disease using the anti-TIM-3 monoclonal antibody or antibody fragment thereof in the present invention

[0277] A disease relating to TIM-3 can be diagnosed by detecting or determining TIM-3 or a cell expressing TIM-3 using the monoclonal antibody or antibody fragment of the present invention.

[0278] A diagnosis of cancer which is one of the diseases relating to TIM-3 can be carried out by, for example, the following detection or measurement of TIM-3.

[0279] The diagnosis of cancer can be carried out by detecting TIM-3 expression on the cell in a patient's body by an immunological method such as a flow cytometry.

[0280] An immunological method is a method in which an antibody amount or an antigen amount is detected or determined using a labeled antigen or antibody. Examples of the immunological method include radioactive substance-labeled immunoantibody method, enzyme immunoassay, fluorescent immunoassay, luminescent immunoassay, Western blotting method, physico-chemical means and the like.

[0281] Examples of the radioactive substance-labeled immunoantibody method include a method, in which the antibody or antibody fragment of the present invention is allowed to react with an antigen, a cell expressing an antigen or the like, then anti-immunoglobulin antibody subjected to a radioactive labeling or a binding fragment thereof is allowed to react therewith, followed by determination using a scintillation counter or the like.

[0282] Examples of the enzyme immunoassay include a method, in which the antibody or antibody fragment of the present invention is allowed to react with an antigen, a cell expressing an antigen or the like, then an anti-immunoglobulin antibody or an binding fragment thereof subjected to antibody labeling is allowed to react therewith and the colored pigment is measured by a spectrophotometer, and, for example, sandwich ELISA may be used.

[0283] As a label used in the enzyme immunoassay, any known enzyme label (Enzyme Immunoassay, published by Igaku Shoin, 1987) can be used as described previously. Examples include alkaline phosphatase labeling, peroxidase labeling, luciferase labeling, biotin labeling and the like.

[0284] Sandwich ELISA is a method in which an antibody is bound to a solid phase, antigen to be detected or measured is trapped and another antibody is allowed to react with the trapped antigen. In the ELISA, two kinds of antibody which recognizes the antigen to be detected or measured or the antibody fragment thereof in which antigen recognizing site is different are prepared and the first antibody or antibody fragments is previously adsorbed on a plate (such as a 96-well plate) and the second antibody or antibody fragment is labeled with a fluorescent substance such as FITC, an enzyme such as peroxidase, or biotin.

[0285] The plate to which the above antibody is adsorbed is allowed to react with the cell separated from living body or disrupted cell suspension thereof, tissue or disintegrated solution thereof, supernatant of cell culture, serum, pleural effusion, ascites, eye solution or the like, then allowed to react with a labeled monoclonal antibody or an antibody fragment and a detection reaction corresponding to the labeled substance is carried out. The antigen concentration in the sample to be tested can be calculated from a calibration curve prepared by a stepwise dilution of antigen of known concentration.

[0286] As antibody used for sandwich ELISA, any of polyclonal antibody and monoclonal antibody may be used or antibody fragments such as Fab, Fab' and $F(ab)_2$ may be used. As a combination of 2 kinds of antibodies used in sandwich ELISA, a combination of monoclonal antibodies or antibody fragments recognizing different epitopes may be used or a combination of polyclonal antibody with monoclonal antibody or antibody fragments may be used.

[0287] A fluorescent immunoassay includes a method described in the literatures [Monoclonal Antibodies - Principles and practice, Third Edition, Academic Press (1996*);* Manual for Monoclonal Antibody Experiments, Kodansha Scientific (1987)] and the like. As a label for the fluorescent immunoassay, any of known fluorescent labels [Fluorescent Immunoassay, by Akira Kawao, Soft Science, (1983)] may be used. Examples of the label include FITC, RITC and the like.

[0288] The luminescent immunoassay can be carried out using the methods described in the literature [Bioluminescence and Chemical Luminescence, Rinsho Kensa, 42, Hirokawa Shoten (1998)] and the like. As a label used for luminescent immunoassay, any of known luminescent labels can be included. Examples include acridinium ester, lophine or the like may be used.

[0289] Western blotting is a method in which an antigen or a cell expressing an antigen is fractionated by SDS (Sodium dodecyl sulfate)-PAGE [Antibodies-A Laboratory Manual (Cold Spring Harbor Laboratory, 1988)], the gel is blotted onto PVDF membrane or nitrocellulose membrane, the membrane is allowed to react with antigen-recognizing antibody or

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antibody fragment, further allowed to react with an anti-mouse IgG antibody or antibody fragment which is labeled with a fluorescent substance such as FITC, an enzyme label such as peroxidase, a biotin labeling, or the like, and the label is visualized to confirm the reaction. An example thereof is described below.

[0290] Cells or tissues in which a polypeptide having the amino acid sequence represented by SEQ ID NO: 53 is expressed are dissolved in a solution and, under reducing conditions, 0.1 to 30 μg as a protein amount per lane is electrophoresed by an SDS-PAGE method. The electrophoresed protein is transferred onto a PVDF membrane and allowed to react with PBS containing 1 to 10% of BSA (hereinafter referred to as "BSA-PBS") at room temperature for 30 minutes for blocking.

[0291] Here, the monoclonal antibody of the present invention is allowed to react therewith, washed with PBS containing 0.05 to 0.1% Tween 20 (hereinafter referred to as "Tween-PBS") and allowed to react with goat anti-mouse IgG labeled with peroxidase at room temperature for 2 hours. It is washed with Tween-PBS and a band to which the monoclonal antibody is bound is detected using ECL Western Blotting Detection Reagents (manufactured by Amersham) or the like to thereby detect a polypeptide having the amino acid sequence represented by SEQ ID NO: 1. As an antibody used for the detection in Western blotting, an antibody which can be bound to a polypeptide having no three-dimensional structure of a natural type is used.

[0292] The physicochemical method is specifically carried out by reacting TIM-3 as the antigen with the antibody or antibody fragment of the present invention to form an aggregate, and detecting this aggregate. Other examples of the physicochemical methods include a capillary method, a one-dimensional immunodiffusion method, an immunoturbidimetry, a latex immunoturbidimetry [Handbook of Clinical Test Methods, Kanehara Shuppan, (1988)] and the like.

[0293] For example, in a latex immunodiffusion method, a carrier such as polystyrene latex having a particle size of about of 0.1 to 1 μm sensitized with antibody or antigen may be used and when an antigen-antibody reaction is carried out using the corresponding antigen or antibody, scattered light in the reaction solution increases while transmitted light decreases. When such a change is detected as absorbance or integral sphere turbidity, it is possible to measure antigen concentration, etc. in the sample to be tested.

[0294] In addition, for the detection of the cell expressing TIM-3, known immunological detection methods can be used, and an immunoprecipitation method, an immuno cell staining method, an immune tissue staining method, a fluorescent antibody staining method and the like are preferably used.

[0295] An immunoprecipitation method is a method in which a cell expressing TIM-3 is allowed to react with the monoclonal antibody or antibody fragment of the present invention and then a carrier having specific binding ability to immunoglobulin such as protein G-Sepharose is added so that an antigen-antibody complex is precipitated. Also, the following method can be carried out. The above-described antibody or antibody fragment of the present invention is solid-phased on a 96-well plate for ELISA and then blocked with BSA-PBS.

[0296] When the antibody is in a non-purified state such as a culture supernatant of hybridoma cell, anti-mouse immunoglobulin or rat immunoglobulin or protein A or Protein G or the like is previously adsorbed on a 96-well plate for ELISA and blocked with BSA-PBS and a culture supernatant of hybridoma cell is dispensed thereto for binding. After BSA-PBS is discarded and the residue is sufficiently washed with PBS, reaction is carried out with a dissolved solution of cells or tissues expressing TIM-3. An immune precipitate is extracted from the well-washed plate with a sample buffer for SDS-PAGE and detected by the above-described Western blotting.

[0297] An immune cell staining method or an immune tissue staining method are a method where cells or tissues in which antigen is expressed are treated, if necessary, with a surfactant, methanol or the like to make an antibody easily permeate to the cells or tissues, then the monoclonal antibody of the present invention is allowed to react therewith, then further allowed to react with an anti-immunoglobulin antibody or binding fragment thereof subjected to fluorescent labeling such as FITC, enzyme label such as peroxidase or biotin labeling and the label is visualized and observed under a microscope.

[0298] In addition, cells can be detected by an immunofluorescent staining method where cells are allowed to react with a fluorescence-labeled antibody and analyzed by a flow cytometer [Monoclonal Antibodies - Principles and practice, Third Edition, Academic Press (1996), Manual for Experiments of Monoclonal Antibodies, Kodansha Scientific (1987)] in which cells are allowed to react with a fluorescence-labeled antibody and analyzed by a flow cytometer.

[0299] Particularly, the monoclonal antibody or antibody fragment of the present invention which binds to amino acids sequences of an extracellular region of the TIM-3 or a three-dimensional structure thereof can detect a cell expressing the polypeptide maintaining a natural three-dimensional structure by a fluorescence antibody method.

[0300] In addition, in the case of using FMAT8100HTS system (manufactured by Applied Biosystems) and the like among fluorescent antibody staining methods, the antigen quantity or antibody quantity can be measured without separating the formed antibody-antigen complex and the free antibody or antigen which is not concerned in the formation of the antibody-antigen complex.

The present invention can provide a monoclonal antibody or an antibody fragment of thereof, which binds to amino acids sequences of an extracellular region of TIM-3 or a three-dimensional structure and expresses ADCC activity; a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant

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obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a therapeutic agent or a diagnostic agent comprising the antibody or the antibody fragment thereof as an active ingredient.

[0301] Hereinafter, exemplary embodiments of the present invention will be described specifically. However, the present invention is not limited to the exemplary embodiments described below.

Example

[Example 1]

(Molecular cloning of human TIM-3 cDNA and establishment of stable expression cell)

[0302] Human TIM-3 cDNA was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and Human MTC panel (manufactured by Clontech) as a template. For primers, TIM-3 Fw2 (SEQ ID NO: 31) and TIM-3 Re2 (SEQ ID NO: 32) were used. The obtained PCR product was inserted into pGEM-T Easy vector (manufactured by Promega Corp.), and the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify. The plasmid DNA was extracted by the miniprep method.

[0303] By analyzing the sequence of purified DNA using primers TIM-3 Fw and TIM-3 Re2, the clone having the same sequence as the coding region of GenBank accession number NM_032782 was selected. A human TIM-3 retrovirus (hTIM-3/pMCs-IG) was constructed by recombination of the insert of the selected clone into pMCs-IRES-GFP vector (manufactured by Cell Biolabs Inc.) using Retrovirus Constructive System Ampho (manufactured by Takara Bio Inc.).

[0304] The human TIM-3 retrovirus was infected into Jurkat cell (ATCC accession NO: CRL-2063), EoL-1 cell (RIKEN CELL BANK NO: RCB0641) and L929 cell (manufactured by Sigma-Aldrich Co., LLC.) which were confirmed not to endogenously express TIM-3 was confirmed by flow cytometry analysis. After several passages, TIM-3 positive cells were collected by FACSAria (manufactured by BD Biosciences). Human TIM-3 stable expression cell line was established by recollecting the TIM-3 positive cells by FACSAria after several passages.

[Example 2]

(Preparation of expression vector of fusion protein of soluble extracellular human TIM-3-human Fc)

[0305] The cDNA encoding the extracellular region of human TIM-3 was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and hTIM-3/pMCs-Ig constructed in Example 1 as a template. For primers, pMCs-Fw (SEQ ID NO: 33) and TIM3ED-FcReXba 2 (SEQ ID NO: 34) were used.

[0306] The amplified PCR product was inserted into pTracer-CMV-FLAG-human Fc vetctor [modified vector in which FLAG and Fc domain of human IgG1 were inserted into pTracer-CMV (manufactured by Invitrogen) vector between *Xba*I site and *Apa*I site]. After the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify, the plasmid DNA (sTIM-3-FLAG-Fc/pTracerCMV) was extracted by miniprep method.

[0307] By the analysis of the DNA sequence using T7 (SEQ ID NO: 35) and hTIM-3 Fw1 (SEQ ID NO: 36) as a primer, the purified sTIM-3-FLAG-Fc/pTracerCMV was confirmed to have the same nucleotide sequence as the corresponding region in GenBank accession number NM_032782. The nucleotide sequence (from *EcoR*I recognition site to *Apa*I recognition site) was identical to the nucleotide sequence represented by SEQ ID NO: 37.

[Example 3]

(Preparation of expression vector of soluble extracellular human TIM-3)

[0308] The cDNA encoding the extracellular region of human TIM-3 was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and sTIM-3-FLAG-Fc/pTracerCMV established in Example 2 as a template. As primers, TIM-3 Fw2 (SEQ ID NO: 31) and TIM3ED-FLAG4aa (SEQ ID NO: 39) were used.

[0309] Next, FLAG epitope was ligated by PCR method using ExTaq (manufactured by Takara Bio Inc.), TIM-3 Fw2 (SEQ ID NO: 31) and C-FLAG-NotR2 (SEQ ID NO: 40) as primers and the obtained PCR product as a template. The PCR product was inserted into pGEM-T Easy vector (manufactured by Promega Corp.). After the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify the plasmid DNA (sTIM-3-FLAG/pEF6Myc_HisC) was extracted by the miniprep method.

[0310] By the DNA sequence analysis using T7 (SEQ ID NO: 35) and BGH-R (SEQ ID NO: 41) as a primer, the purified sTIM-3-FLAG-Fc/pEF6 Myc_HisC was confirmed to have the same nucleotide sequence as the corresponding region in GenBank accession number NM_032782.

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[Example 4]

(Preparation of the fusion protein of soluble extracellular human TIM-3-human Fc and soluble extracellular human TIM-3)

[0311] Each of sTIM-3-FLAG-Fc/pTracerCMV plasmid DNA and sTIM-3-FLAG-Fc/pEF6 Myc_HisC plasmid DNA obtained in Example 2 and Example 3, respectively, was introduced into HEK293F cells (manufactured by Invitrogen) and was transiently expressed. Six days after, each of the cell supernatant was recovered and used for protein purification.

[0312] The culture supernatant containing the fusion protein of soluble extracellular human TIM-3-human Fc or soluble extracellular human TIM-3 was recovered by centrifugation six days after transfection. The fusion proteins were purified by an anti-FLAG column prepared using Anti-FLAG M2 Agarose Affinity gel (manufactured by Sigma-Aldrich Co., LLC.) and by FLAG peptides (manufactured by Sigma-Aldrich Co., LLC.) according to the manufacturer's protocols.

[0313] The eluted samples were fractionated and then each fraction was analyzed by SDS-PAGE under reduced conditions, and then silver staining and Western blotting were carried out. For Western blotting, an anti-FLAG M2 antibody (manufactured by Sigma-Aldrich Co., LLC.) and an alkaline phosphatase labeled rabbit anti-mouse immunoglobulin antibody was used (manufactured by Dako). The fraction containing the target protein was concentrated using Amicon Ultra 4 10K (manufactured by Millipore) and was applied for gel filtration chromatography using Superdex 200 gp column (manufactured by GE Healthcare).

[0314] After fractionation, each fraction was applied to SDS-PAGE under reduced condition, silver stained and then Western blotted. The fraction in which the target protein was found was concentrated and washed with 0.5 ml of PBS. The soluble extracellular human TIM-3-human Fc fusion protein or soluble extracellular human TIM-3 was collected after filtering through the MILLEX-GV sterilizing filter (manufactured by Millipore) having a pore diameter of 0.22 μm. The purity of the protein was measured by Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) which is a kit to detect endotoxin and confirmed that the fraction was sufficiently purified.

[Example 5]

(Preparation of human antibody expressing mouse)

[0315] The mice used for immunization has a genetic background in which both of endogenous Ig heavy chain locus and κ light chain locus were homozygously disrupted and maintained a chromosome 14 fragment (SC20) comprising a human heavy chain transchromosome, and a human Igκ chain transgene (KCo5) at the same time. This mouse strain was established by cross breeding mice strain A which had human Ig heavy chain loci and mice strain B which maintained human Igκ chain transgene.

[0316] Mouse strain A is a homozygote in which both of the endogenous Ig heavy chain locus and κ light chain locus are homozygously disrupted, and maintains a chromosome 14 fragment (SC20) which can be transmitted to the progeny and for example, described in Tomizuka *et al.* (Tomizuka, K. et al., 2000, Proc. Natl. Acad. Sci. U.S.A. 97:722-727).

[0317] Mouse Strain B is a mouse strain (transgenic mouse) in which both of the endogenous Ig heavy chain locus and κ light chain locus are homozygously disrupted, and maintains a human Igκ light chain transgene (KCo5), and is ,for example, described in Fishwild et al. (1996) [Nature Biotechnology 14:845-851].

[0318] The animal individual was obtained by cross-breeding male mouse of strain A and female mouse of strain B, or female mouse of strain A and male mouse of strain B and was a mouse in which human Ig heavy chain and Igκ light chain could be detected at the same time in their serum [Ishida& Lonberg, IBC's 11th Antibody Engineering, Abstract 2000] was further used for immunological experiments. For your information,, the human antibody expressing mouse (hereinafter referred to as "KM mouse") can be obtained from Kyowa Hakko Kirin by establishing a license.

[Example 6]

(Preparation of anti-human TIM-3 monoclonal antibody)

[0319] The preparation of monoclonal antibody in the present Example was carried out according to the general methods, such as published in Monoclonal Antibody Experiment Manual, (Yasuhigashi Tamie et al., Kodansha, 1991). Either TIM-3 expressing L929 cell prepared in Example 1 or the soluble extracellular human TIM-3-human Fc fusion protein prepared in Example 4 was used as a TIM-3 immunogen. The immunized animal was KM mouse described above.

[0320] The TIM-3 expressing L929 cell was intraperitoneally injected into mice at a dose of 1×10^7 cells/animal. After the first immunization, the same cell was further administered for another three times or more thereafter. Three days before obataining the spleen, the soluble extracellular human TIM-3-human Fc fusion protein prepared in Example 4 was administered from caudal vein at 20 μg/mouse. The spleen was surgically removed from the immunized mouse, put into 4 ml of PBS and mashed on a mesh (cell strainer: manufactured by Falcon) using a syringe piston.

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[0321] The cells were precipitated by centrifuging the cell suspension which was passed through the mesh, and the cells were resupsended in 1 ml of Red Blood Cell Lysis Buffer (manufactured by Sigma-Aldrich Co., LLC.). After incubating for 5 min at room temperature, 10 ml of serum free DMEM medium (manufactured by Invitrogen) (hereinafter referred to as "serum free DMEM medium") containing 50 unit/ml penicillin and 50 μg/ml streptomycin was added, and the cells were precipitated by centrifugation.

[0322] The cell pellets were resuspended in serum free DMEM medium. On the other hand, myeloma cell line SP2/0 (ATCC NO: CRL-1581) was cultured in DMEM medium containing 10% FBS, 50 unit/ml penicillin and 50 μg/ml streptomycin (hereinafter referred to as "DMEM medium containing serum") and incubated at 37°C under 5% CO_2 so as not exceed the cell number of 1×10^6 cells/ml.

[0323] The SP2/0 cells were washed with 10 ml of serum free DMEM medium and suspended in serum free DMEM medium as in the case of the spleen derived cells. These suspension of spleen derived cells and suspension of myeloma cell were mixed at a ratio of 5:1, centrifuged, and the supernatant was completely removed. To fuse the cells, 1 ml of 50% (w/v) of polyethylene glycol (manufactured by Boehringer Mannheim Corp.) was slowly added while stirring the pellet with the pipette tip, and then 1 ml of serum free DMEM medium preheated at 37°C was added. Slowly, 5 ml and 10 ml of serum free DMEM was added followed by 5 min incubation at 37°C under 5% CO_2.

[0324] After the centrifugation and removal of supernatant, the fused cells were resuspended in 50 ml of DMEM medium containing 10% FBS (manufactured by Invitrogen), penicillin-streptomycin-glutamine (manufactured by Invitrogen, 100-fold dilution of Catalog No.10378-016), IL-6 (5 ng/ml), and 2-mercaptoethanol (manufactured by Invitrogen, 1000-fold dilution of Catalog No. 21985-023) (hereinafter referred to as "DMEM medium containing IL-6") and incubated at 37°C under 5% CO_2.

[0325] The next day, cells were harvested by pipetting, centrifuged and the pellet was resuspended in 10 ml of DMEM medium containing IL-6. On the next day, Hypoxanthine-aminopterin-thymidine (hereinafter referred to as "HAT", manufactured by Sigma-Aldrich Co., LLC.) was added. After incubating for about 7 to 10 days, the culture supernatant was collected for hybridoma screening.

[Example 7]

(Preparation of anti-human TIM-3 mouse monoclonal antibody)

[0326] The preparation of monoclonal antibody in the present Example was carried out according to the general methods published in Introduction to Monoclonal Antibody Experiment (Yasuhigashi Tamie et al., Kodansha, 1991). Either TIM-3 expressing L929 cell prepared in Example 1 or soluble extracellular human TIM-3 prepared in Example 4 was used as a TIM-3 immunogen. The immunized animal was Balb/c mouse (purchased from Nihon Charles River).

[0327] First, a Balb/c mouse was immunized by injecting TIM-3 expressing L929 cells intraperitoneallyat a dose of 1×10^7 cells/animal. After the first immunization, the same cell was further administered for another three times or more thereafter. Three days before extracting the spleen, the soluble extracellular human TIM-3 protein prepared in Example 4 was administered from caudal vein at 20 μg/mouse. The spleen was surgically removed from the immunized mouse, put into 4 ml of PBS and mashed on a mesh (cell strainer: manufactured by Falcon) using a syringe piston.

[0328] The cells were precipitated by centrifuging the cell suspension which was passed through the mesh, and the cells were resuspended in 1 ml of Red Blood Cell Lysis Buffer (manufactured by Sigma-Aldrich Co., LLC.). After incubating for 5 min at room temperature, 10 ml of serum free DMEM medium was added, and then cells were precipitated by centrifugation. The pellets were resuspended in serum free DMEM medium. On the other hand, myeloma cell line SP2/0 (ATCC NO: CRL-1581) were cultured in DMEM medium by incubating at 37°C under 5% CO_2 to the cell number of 1×10^6 cells/ml or less.

[0329] Similar to the spleen derived cells, the SP2/0 cells were washed with 10 ml of serum free DMEM medium and suspended in serum free DMEM medium. The suspension of spleen cell and the suspension of myeloma cell were mixed at a ratio of 5:1. After centrifugation, the supernatant was removed. To this pellet, 1 ml of 50% (w/v) of polyethylene glycol (manufactured by Boehringer Mannheim Corp.) was slowly added while stirring the pellet with the pipette tip, and then 1 ml of serum free DMEM medium preheated to 37°C was added.

[0330] To this suspension, 5 ml of DMEM medium was slowly added. After further adding 10 ml of serum free DMEM, the suspension was incubated for 5 min at 37°C under 5% CO_2. After the suspension was centrifuged, the cell pellets were resuspended in 50 ml of DMEM medium containing IL-6 and cultured at 37°C under 5% CO_2. After incubation for one day, cells were collected by pipetting. After centrifugation, the pellet was resuspended in 10 ml of DMEM medium containing IL-6. On the next day, HAT medium (manufactured by Sigma-Aldrich Co., LLC.) was added. After incubation for about 7 to 10 days, the culture supernatant was recovered and used for hybridoma screening.

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[Example 8]

(Screening of hybridoma producing a human or mouse monoclonal antibody which binds to human TIM-3)

[0331] The hybridoma was screened using the cell supernatant prepared in Example 6 and Example 7. The method to be used was a flow cytometry method using human TIM-3 expressing cell lines. First, the human TIM-3 expressing Jurkat cell or EoL-1 cell prepared in Example 1 were washed with staining medium (PBS containing 2% FBS and 0.05% sodium azide), and then resuspended in 1 ml of staining medium to give a cell density of 1×10^6 cells/ml. The cell suspension was dispensed at 10 μl/well into a 96-well culture plate.

[0332] Then, 50 μl of the hybridoma supernatant was added and incubated for 30 min at 4°C. After the cells were washed twice with the staining medium, 50 μl of labeled antibody which was diluted 200-fold with staining medium was added to each well and incubated for 30 min at 4°C. As for labeled antibodies, Goat $F(ab')_2$ Anti-Human IgG (γ chain specific)-R-PE (manufactured by SouthernBiotech) were used for the human monoclonal antibody and Goat $F(ab')_2$ Anti-Mouse IgG(H+L)R-PE (manufactured by SouthernBiotech) was used for the mouse monoclonal antibody.

[0333] After washing twice with staining medium, the cells were analysed using FACSCalibur (manufactured by BD Biosciences) as a primary screening. After positive clones were picked and expanded, the hybridoma cells were clone-sorted by FACSAria (manufactured by BD Biosciences) and cultured for seven days in DMEM medium containing IL-6 which further contains Hypoxanthine-thymidine (hereinafter referred to as "HT", manufactured by Sigma-Aldrich Co., LLC.).

[0334] Harvested supernatants were screened as the same as in the primary screening method for cloning of hybridomas expressing anti-human TIM-3 human monoclonal antibody and hybridomas expressing anti-human TIM-3 mouse monoclonal antibody and selected hybridoma clone was used for purification of monoclonal antibody.

[Example 9]

(Purification of hybridoma derived monoclonal antibody)

[0335] An anti-TIM-3 human or mouse monoclonal antibody was purified from the anti-human TIM-3 human or mouse monoclonal antibody expressing hybridoma prepared in Example 8. Briefly, hybridoma supernatant containing a high concentration of the anti-TIM-3 antibody was prepared using a CELLine antibody production system (manufactured by BD Biosciences) and the anti-human TIM-3 monoclonal antibody was purified from the supernatant. First, to adapt the cloned hybridoma to the serum free medium, the hybridomas were cultured for several days in a medium mixing DMEM medium containing HT and IL-6 and BD Cell MAb serum free medium (manufactured by BD Biosciences) at a ratio of 1:1, and then cultured for several days in a medium having a mixing ratio of 1:2.

[0336] Next, the hybridomas were cultured in a BD Cell MAb serum free medium (manufactured by BD Biosciences) and adapted hybridomas to the serum free medium. The hybridoma cells adapted to the serum free medium were cultured in CL-1000 flasks according to the manufacturer's instructions and the supernatant of the hybridoma containing high concentration of the anti-TIM-3 antibody was collected from the flasks. The antibody from the supernatant of the hybridoma was purified in accordance with the standard procedures using Protein A.

[0337] Specifically, MabSelect (manufactured by GE Healthcare) were packed in an open column and the supernatant diluted 2-fold by PBS was loaded and then eluted with 0.1 mol/L Glycine-HCl (pH 2.7), followed by concentration with Amicon Ultra (manufactured by Millipore). Next, by using a NAP-5 column (manufactured by GE Healthcare), the obtained solution was equilibrated with PBS buffer, and then sterilized by filtration to obtain 4 clones of the anti-human TIM-3 human monoclonal antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) and one clone (antibody 8213) of the anti-human TIM-3 mouse monoclonal antibody.

[Example 10]

(Identification of isotype of anti-TIM-3 human monoclonal antibody)

[0338] Each of the isotype of anti-TIM-3 human monoclonal antibodies which was prepared in Example 9 was determined by a solid-phase ELISA and flow cytometry.
Specifically, as for the solid-phase ELISA, soluble extracellular human TIM-3 obtained by Example 4 was diluted with a Carbonate-Biacarbonate Buffer (manufactured by Sigma-Aldrich Co., LLC.) to give a concentration of 1 μg/ml and dispensed at 50 μl/well into a 96-well culture plate (Maxisorp, manufactured by Nunc). The soluble extracellular human TIM-3 was adsorbed to the microplate by incubating overnight at 4°C.

[0339] After the supernatant was discarded, SuperBlock Blocking Buffer in TBS (manufactured by PIERCE) was added to the plate and then incubated for 10 min at room temperature and 50 μl of the culture supernatant of the hybridoma

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was added and then incubated for 30 min at room temperature. After washing each well with Tris-buffered saline containing 0.1% Tween20 (TBS-T), 50 μl of each of HRP-labeled mouse anti-human IgG1, anti-human IgG2, anti-human IgG3 or anti-human IgG4 antibodies, each antibody was diluted 2000, 2000, 2000 and 4000-fold with TBS-T containing 10% SuperBlock Blocking Buffer, respectively; manufactured by SouthernBiotech), were added and incubated for 30 minutes at room temperature.

[0340] After washing each well with TBS-T, 50 μl of substrate buffer (TMB, manufactured by DAKO) was added and then incubated for 20 min at room temperature. The reaction was stopped by adding 50 μl of 0.5 mol/L sulfuric acid (manufactured by Wako Pure Chemical Industries, Ltd.). The absorbance at wavelength of 450 nm (reference wavelength: 570 nm) was measured on a microplate reader (VersaMax, manufactured by Molecular Devices, LLC.).

[0341] In addition, in flow cytometry method, the Jurkat cell which expressed human TIM-3 prepared in Example 1 was washed with staining medium, and then 50 μl of the supernatant of the hybridoma was added and incubated for 30 min at 4°C. After washing, 50 μl of each of Mouse anti-Human IgG1-PE, Mouse anti-Human IgG2-PE, Mouse anti-Human IgG3 (Hinge)-PE and Mouse anti-Human IgG4(Fc)-PE (manufactured by SouthernBiotech) which was diluted 100-fold with staining medium was added, allowed to stand still for 30 min at 4°C and then analyzed using FACSCalibur flow cytometer (manufactured by BD Biosciences).

[0342] Based on the result, the isotypes of antibody 512, antibody 644, and antibody 4545 were IgG1, IgG4 and IgG2, respectively.

[Example 11]

(Identification of the isotype of the mouse monoclonal antibody for human TIM-3)

[0343] The isotype of the anti-human TIM-3 mouse monoclonal antibody prepared in Example 9 was determined using Iso Strip Mouse Monoclonal Antibody Isotyping Kit (manufactured by Roche-diagnostics). By using the supernatant of the hybridoma containing the anti-TIM-3 antibody prepared in Example 8, the isotype was determined according to the manufacturer's instructions. As a result, the isotype of antibody 8213 was determined as IgG2b.

[Example 12]

(Isolation of anti-TIM-3 monoclonal antibody gene)

[0344] The gene of anti TIM-3 human or mouse monoclonal antibody which was prepared in Example 9 was isolated from the hybridoma which produced anti-human TIM-3 human or mouse monoclonal antibody prepared in Example 9. Total RNA was extracted from the cloned hybridoma using High Pure RNA Isolation Kit (manufactured by Roche-Diagnostics), according to the manufacturer's instructions. The cloning of the cDNA encoding the variable region was carried out using SMART RACE cDNA amplification Kit (manufactured by Clontech) in accordance with the instructions attached thereto.

[0345] The heavy chain (VH) of the human monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hh-6 (SEQ ID NO: 44). Using part of the reaction product as a template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hh-3 (SEQ ID NO: 45). The cloning of the PCR product was carried out using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R primer) and the amino acid sequence was deduced from the nucleotide sequence.

[0346] As a result, the nucleotide sequence and the amino acid sequence of VH of antibody 512 were represented by SEQ ID NO: 54, was and SEQ ID NO: 55, respectively, the nucleotide sequence and the amino acid sequence of VH of antibody 644 were represented by SEQ ID NO: 58 and SEQ ID NO: 59, respectively, the nucleotide sequence and the amino acid sequence of VH of antibody 4545 were represented by SEQ ID NO: 7 and SEQ ID NO: 8, respectively, and the nucleotide sequence and the amino acid sequence of VH of antibody 4177 were represented by SEQ ID NO: 17 and SEQ ID NO: 18, respectively. Each of the obtained variable regions obtained and the constant region of human IgG1 were inserted and ligated into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0347] Human monoclonal antibody light chain (VL) was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hk-2 (SEQ ID NO: 46). Using part of the reaction product as a template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hk-6 (SEQ ID NO: 47). The PCR product was cloned using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0348] As a result, the nucleotide sequence and the amino acid sequence of VL of antibody 512 were represented by SEQ ID NO: 56 and SEQ ID NO: 57, respectively, the nucleotide sequence and the amino acid sequence of VL of

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antibody 644 were represented by SEQ ID NO: 60 and SEQ ID NO: 61, respectively, the nucleotide sequence and the amino acid sequence of VL of antibody 4545 were represented by SEQ ID NO: 9 and SEQ ID NO: 10, respectively, and the nucleotide sequence and the amino acid sequence of VH of antibody 4177 were represented by SEQ ID NO: 19 and SEQ ID NO: 20, respectively. Each of the obtained variable regions and constant region of κ chain were inserted and ligated into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0349] The heavy chain (VH) of mouse monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and a primer, mH_Rv1 (SEQ ID NO: 48). Using part of the reaction product e as template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mH_Rv2 (SEQ ID NO: 49).

[0350] The cloning of the PCR product was carried out using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0351] As a result, the nucleotide sequence and the amino acid sequence of VH of antibody 8213 were represented by SEQ ID NO: 27 and SEQ ID NO: 28, respectively. The obtained variable region and the constant region of mouse IgG2a were ligated and inserted into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0352] The light chain (VL) of the mouse monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mK_Rv1 (SEQ ID NO: 50). Additionally, part of the reaction product was used as a template, and PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mK_Rv2 (SEQ ID NO: 51). The PCR product was cloned using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). Nucleotide sequences were analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0353] As a result, the nucleotide sequence and the amino acid sequence of the VL of antibody 8213 were represented by SEQ ID NO: 29 and SEQ ID NO: 30, respectively. The variable region obtained and the constant region of κ chain were ligated and inserted into the above mentioned expression vector encoding VH of antibody 8213.

[0354] In addition, in order to obtain anti-dinitrophenyl (DNP) human IgG1 antibody as a negative control, the nucleotide sequence of the heavy chain (VH) of the antibody (SEQ ID NO: 62), the nucleotide sequence of the light chain of the antibody (SEQ ID NO: 64), and the constant region of human IgG1 and the κ chain were inserted into expression vector N5KG1 (manufactured by Biogen IDEC Inc.).

[Example 13]

(Purification of recombinant anti-TIM-3 human monoclonal defucose antibody)

[0355] A cell expressing a recombinant antibody was produced by introducing the recombinant antibody expression vector prepared in Example 12 into the host cell. As the host cell for expression, $FUT8^{-/-}$CHO cells were used in accordance with a published report *(Clin Cancer Res* 2006: 12(9) Iida *et al.*). Briefly, the expression vectors for each of 512, 644, 4545 and 4177 prepared in Example 12 were introduced into $FUT8^{-/-}$ CHO cell using FreeStyle™ MAX Reagent (manufactured by Invitrogen). The $FUT8^{-/-}$CHO cells were cultured on a shaker at 37°C under 5% CO_2. The culture supernatant was recovered after about five days.

[0356] The recovered supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. Antibodies were affinity purified using PBS as the binding buffer and 0.02 mol/L sodium citrate (pH 2.7, 50 mmol/L NaCl) buffer as the elution buffer. The elution fraction was added with 0.2 mol/L of sodium phosphate buffer (pH 7.0).

[0357] The prepared antibody solution was equilibrated with PBS using NAP-25 column (manufactured by GE Healthcare) and concentrated with Amicon Ultra (10000 cut, manufactured by Millipore). After sterilization by filtration, the recombinant anti-TIM-3 human monoclonal defucose antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) were obtained. The purity of the protein was determined by Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that these antibodies were sufficiently purified.

The vector for expressing anti-DNP prepared in Example 12 was introduced into CHO-DG44 cells. The CHO-DG44 cells were cultured on a shaker at 37°C under 5% CO_2. The cultured supernatant was recovered following incubation for 5 days. The collected supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. The affinity purification of the antibodies was carried out using PBS as the binding buffer and 0.02 mol/L of sodium citrate (pH 2.7, 50 mmol/L NaCl) buffer as the elution buffer. The elution fraction was added with 0.2 mol/L of sodium phosphate buffer (pH 7.0). The prepared antibody solution was equilibrated with PBS and concentrated using NAP-25 column (manufactured by GE Healthcare) with Amicon Ultra (10000 cut, manufactured by Millipore) respectively. After sterilization by filtration, the recombinant anti-DNP antibody was obtained. The purity of the protein was determined using a Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that the antibody was sufficiently purified.

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[Example 14]

(Purification of recombinant anti-TIM-3 mouse monoclonal antibody)

[0358] The vector for expressing antibody 8213 prepared in Example 12 was introduced into HEK293F cells (manufactured by Invitrogen) using 293 fectin (manufactured by Invitrogen). HEK293F cells were incubated on a shaker at 37°C under 5% CO_2. The cultured supernatant was collected after incubation for five days. The collected supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. The affinity purification of the antibody was carried out using PBS as a binding buffer and 0.02 mol/L sodium citrate buffer (pH 2.7, 50 mmol/L NaCl) as an elution buffer.

[0359] To the elution fraction, 0.2 mol/L of sodium phosphate buffer (pH 7.0) was added. The prepared antibody solution was equilibrated with PBS using NAP-25 column (manufactured by GE Healthcare) and concentrated with Amicon Ultra (10000 cut, manufactured by Millipore). After sterilization by filtration, the recombinant anti-TIM-3 mouse monoclonal antibody (8213 antibody) was obtained. The purity of the protein was determined using a Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that the antibody was sufficiently purified.

[Example 15]

(Fluorescent labeling of purified monoclonal antibody)

[0360] The fluorescent labeling of the hybridoma derived monoclonal antibody and recombinant monoclonal antibody was carried out by using Alexa Fluor 647 Monoclonal Antibody Labeling Kit (manufactured by Invitrogen) according to the manufacturer's instructions. After the labeling, each antibody was confirmed to be positively labeled with Alexa Fluor 647 (hereinafter referred to as "Alexa-647") by FACSCalibur (manufactured by BD Biosciences) analysis for TIM-3 expressing cells.

[Example 16]

(Classification of epitopes using competition test-1)

[0361] The relation of epitopes between antibodies prepared in Example 13 and Example 14, and commercially available anti-TIM-3 antibody 344823 (Clone 344823, manufactured by R&D Systems) was analyzed by a competition test. In brief, the test antibody was allowed to interact with the TIM-3 expression cell prepared in Example 1, and then whether another anti-TIM-3 antibody could further bind to TIM-3 expressing cells was evaluated by flow cytometry method.

[0362] In the first step, whether or not the competition between each of the monoclonal antibodies prepared in Example 13 and Example 14 (antibody 512, antibody 644, antibody 4177 and antibody 8213) and antibody 34823 were examined. First, human TIM-3 expressing EoL-1 cells and the parent cell, EoL-1 cells were washed with staining medium and then allowed to react with each of the purified subjective monoclonal antibodies (antibody 512, antibody 644, antibody 4177 and antibody 8213) (4°C, 30 min). The final concentration was 10 μg/ml. Next, the PE labeled antibody 344823 (Clone 344823, manufactured by R&D Systems) was added and allowed to react (4°C, 30 min). After washing with staining medium, 7-ADD (manufactured by BD Biosciences) was added and analyzed using FACSCalibur (manufactured by BD Biosciences).

[0363] As a result, the goat derived anti-human TIM-3 polyclonal antibody (manufactured by R&D Systems) used as a positive control and antibody 644 almost completely blocked the binding of PE-labeled antibody 344823 to TIM-3 expressing cell. However, antibody 512, 4545 and 8213 did not inhibit the binding of PE-labeled antibody 344823 to TIM-3 expressing cell. Antibody 4177 inhibited the binding of PE-labeled antibody 344823 to TIM-3 expressing cells, but this inhibition was weaker than that of antibody 644. Accordingly, antibody 644 competed with antibody 344823, but antibodies 512, 4545 and 8213 did not compete with antibody 344823. It was found that the antibody 4177 and antibody 344823 competed with each other, but they competed partially.

[0364] In the second step, among the anti-TIM-3 monoclonal antibodies tested, antibodies 512, 4545 and 8213 which did not interact with antibody 344823 were examined whether or not they competed. The experiment was carried out in the same manner as the first step except for using Alexa-647 labeled antibody 512 or Alexa-647 labeled antibody 8213 prepared in Example 15 as a labeled antibody. As a result, antibody 4545 and antibody 8213 did not inhibit the binding of Alexa-647 labeled antibody 512 to the TIM-3 expressing cells.

[0365] However, antibody 4545 inhibited the binding of Alexa-647 labeled antibody 8213 to TIM-3 expressing cells. Therefore, it was found that antibody 4545 and antibody 8213 competed with each other, but these two antibodies did not compete with antibody 512. This result suggested that antibody 512 recognized different epitope from antibody 344823 and antibody 8213.

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[0366] In the third step, a competitive assay with antibody 512 and antibody 8213 was carried out on antibody 4177 which was shown to partially compete with antibody 344823 in the first step. The experiment was performed in the same manner as described in second step. As a result, antibody 4177 did not inhibit the binding of the Alexa-647 labeled antibody 512 to the TIM-3 expressing cells, but inhibited the binding of the Alexa-647 labeled antibody 8213 to the TIM-3 expressing cells. Therefore, it was found that antibody 4177 did not compete with antibody 512, but only with antibody 8213.

[0367] Based on these results, among the 5 antibodies prepared in Example 13 and Example 14, antibody 644 recognized an epitope which was near the epitope to which antibody 344823 recognized; antibody 512 recognized an epitope different from of the epitope to which antibody 344823 and other four antibodies (antibody 644, antibody 4545, antibody 4177 and antibody 8213) recognized.

[0368] It was suggested that antibody 8213 recognized epitope different from of the epitope to which antibodies 344823 or 512 recognized. It was suggested that antibody 4545 recognized an epitope which was near the epitope to which antibody 8213 recognized. However, antibody 4177 recognized the epitope which was near the epitope to which antibody 8213 recognized, and also weakly recognized an epitope which was near the epitope to which antibody 344823 recognized.

[Example 17]

(Antibody dependent cellular cytotoxicity test of recombinant anti-TIM-3 human monoclonal defucose antibody)

[0369] Regarding the cellular cytotoxic activity via antibody, the ADCC activity against the target cell was measured by using human Peripheral Blood Mononuclear Cells (hereinafter referred to as "PBMC") as an effector in the presence of the antibody. First, peripheral blood was collected from the healthy human donor and mixed with anticoagulant. The obtained blood was loaded onto Ficoll-Plaque Plus (manufactured by GE Healthcare), and centrifuged at 2000 rpm for 20 min using a large scale centrifuge (CF9RX, manufactured by Hitachi Koki Co., Ltd.) so as not to disturb the boundary.

[0370] The middle fraction containing cells were collected and washed with PBS, and then centrifuged at 900 rpm for 20 min to remove the platelets. Thus obtained PBMC was used for the measurement. Next, the Daudi cell used as a target cell and the PBMC were mixed at the ratio of effector/target = 50.

[0371] Each of the recombinant anti-human monoclonal defucose antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) prepared in Example 13 and Example 14, and anti-DNP antibody as a human IgG1 control was suspended in the medium to give a final concentration of 1 μg/ml and a total volume of 100 μl. After mixing, the solution was incubated at 37°C under 5% CO_2 for 4 hr. The lysis ratio of the target cell was evaluated based on the amount of LDH released into the medium. The LDH activity and lysis ratio were calculated using CytoTox 96 Non-Radioactive Cytotoxicity Assay (manufactured by Promega Corp.) in accordance with the instructions attached thereto. As statistical analysis, standard Student's t-test was used wherein a sample having a risk rate (p) of less than 0.05 was considered as one which was significantly different.

[0372] As the result that the ADCC activity on Daudi cells was measured, it was found that antibody 4545 and antibody 4177 exhibited the significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody. This result suggests that anti-TIM-3 mouse antibody 8213 and anti-TIM-3 antibody which competes with anti-TIM-3 mouse antibody 8213 have high ADCC activity.

[0373] Similarly, the ADCC activity was measured by using recombinant anti-TIM-3 human monoclonal defucose antibodies (antibody 4545 and antibody 4177) prepared in Example 13, commercially available anti-TIM-3 monoclonal antibody 344823, and anti-DNP antibody as a negative control.

[0374] First, peripheral blood was collected from the healthy human donor and mixed with anticoagulant. The obtained blood was then loaded onto Ficoll-Plaque Plus (manufactured by GE Healthcare), and centrifuged at 2000 rpm for 20 min using a large scale centrifuge (CF9RX, manufactured by Hitachi Koki Co., Ltd.) so as not to disturb the boundary. The middle fraction containing cells were collected and washed with PBS, and then centrifuged at 900 rpm for 20 min to remove the platelets. Thus obtained PBMC fraction was used for the measurement of t the ADCC activity.

[0375] Next, the Daudi cell used as a target cell and the PBMC were mixed at the ratio of effector/target = 25. Each of the antibody 4545, antibody 4177, antibody 344823, anti-DNP antibody and PBS were suspended in the medium to give a final concentration of 1 μg/ml and a total volume of 100 μl. After mixing, the solution was incubated at 37°C under 5% CO_2 for 4 hr. The lysis ratio of the target cell was evaluated based on using the amount of LDH released into the medium.

[0376] The LDH activity and the lysis ratio were calculated using CytoTox 96 Non-Radioactive Cytotoxicity Assay (manufactured by Promega Corp.) in accordance with the instructions attached thereto. As statistical analysis, standard Student's t-test was used wherein a sample having a risk rate (p) of less than 0.05 was considered as one which was significantly different. As the result of the ADCC activity on Daudi cells was measured, it was found that antibody 4545 and antibody 4177 exhibited the significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody.

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On the other hand, antibody 344823 exhibited no significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody and PBS.

[Example 18]

(Calculating the bound/dissociation constant using anti-TIM-3 antibody)

[0377] The bound/dissociation constant was analyzed using surface plasmon resonance based biosensor (Biacore, manufactured by GE Healthcare). In brief, an anti-human antibody or an anti-mouse antibody was immobilized on the CM5 sensor chips. Next, the anti-TIM-3 human or mouse antibody were made to flow to bind the chip, and then soluble extracellular human TIM-3 prepared in Example 4 was made to flow to examine the bound/association constant of TIM-3 to the anti-TIM-3 antibody. Throughout the experimental process, basically the experimental method provided by GE Healthcare for calculating bound/dissociation constant was used.

[0378] Specifically, CM5 was used as the sensor chip (Research grade, manufactured by GE Healthcare). First, CM5 chip was activated by flowing through a mixed solution of equal parts of 400 mmol/L EDC (N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrocholoride) and 100 mmol/L NHS(N-hyderoxysuccinimide). Next, the anti-human antibody attached to the Human Antibody Capture Kit (manufactured by GE Healthcare) was diluted with the solution attached to the kit and the obtained solution was made to flow so that the required amount of the antibody to an antibody for a human antibody was immobilized on the CM5 chip.

[0379] For the mouse antibody, the antibody against mouse antibody was immobilezed on the CM5 surface by diluting in the solution, which was provided in the Mouse Antibody Capture Kit (manufactured by GE Healthcare antibody). The activated chip surface was blocked by the passing through 1 mol/L for ethanolamide hydrochloride and inactivated.

[0380] Next, one kind of the anti-TIM-3 antibodies was diluted with HBS-EP buffer (manufactured by GE Healthcare) per one flow cell and passed through so as to bind the antibody for the human antibody or antibody for the mouse antibody immobilized on the chip surface. Then soluble extracellular human TIM-3 was flown through. To dissociate the bound anti-TIM-3 antibody from the soluble extracellular human TIM-3, either the total volume of 3 mol/L of $MgCl_2$ which was attached to the Human Antibody Capture Kit or Glycine-HCl (pH 1.7) which was attached to the Mouse Antibody Capture Kit were flown through the cell.

[0381] The above described procedures were considered as one step, and the similar procedure was repeated using different concentrations of the soluble extracellular human TIM-3, to collect data (sensorgram) for calculating the bound/association constant. The concentration of soluble extracellular human TIM-3 used as an analyte was calculated by measuring the absorbance at 280 nm and then converting the value of 1.4 OD to 1 mg/mL.

[0382] The molecular weight of the soluble extracellular human TIM-3 was calculated as 42.8 kDa based on the mobility on electrophoresis. The data was analyzed using Biaevaluation soft-ware (manufactured by GE Healthcare) according to the Biaevaluation Software Handbook. Specifically, the simultaneous Kinetics analysis was performed and the association rate constant (Ka) and dissociation rate constant (Kd) was calculated, by basically adopting a 1:1 Langmuir Binding reaction model was used for fitting. Then the value of the dissociation constant (K_D) was calculated from Kd/Ka.

[0383] Most of the anti-human TIM-3 human monoclonal antibody had the K_D value of the order of 10^{-9} mol/L. In this aspect, there was no correlation between ADCC activity and binding affinity targeting for TIM-3.

[0384] When the anti-human TIM-3 monoclonal antibody was monitored (e.g., monitored the dissociation state for 30 min), there was no dissociation detected with the antigen soluble extracellular human TIM-3 as an antigen. As a result, it was found that it was possible to produce a monoclonal antibody having a very high affinity for human TIM-3.

[Example 19]

(Classification of epitopes using competition test-2)

[0385] The epitopes of anti-TIM-3 antibodies, i.e., antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823, antibody F38-2E2 and anti-DNP antibody as a control were analyzed by a competitive assay. In brief, the unlabeled target antibody was allowed to interact with the TIM-3 expression cell prepared in Example 1, and whether or not other fluorescence labeled anti-TIM-3 antibody was further bound to TIM-3 expression cells were evaluated by flow cytometry method.

[0386] Human TIM-3 expressing EoL-1 cells were washed with staining medium and allowed to react with purified test monoclonal antibodies [antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 (manufactured by R&D Systems), antibody F38-2E2 (manufactured by Imgenex)] (4°C, 30 min). The final concentration was 10 μg/ml. Next, the Alexa-647 or APC labeled anti-TIM-3 antibodies [antibody 344823 (manufactured by R&D Systems) and antibody F38-2E2 (manufactured by eBiosciences)] were added and were allowed to react (4°C, 30 min). After washing with staining medium, 7-ADD (manufactured by BD Biosciences) was added before analyzing with FACSCalibur (man-

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ufactured by BD Biosciences).

[0387] The results are shown in Table 1. As shown in Table 1, the case the degree of the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was similar to that of a negative control were indicated as "+", the case where the degree to the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was found but was lower than that of a negative control was indicated as "+/-"and the case where the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was not detected, it was indicated as "-".

[0388]

[Table 1]

		Unlabeled antibody						
		DNP	512	644	4545	8213	344823	F38-2E2
Alexa-647 or APC labeled antibody	DNP	-	-	-	-	-	-	-
	512	+	-	+	+	+	+	+
	644	+	+	+/-	+	+	+/-	+/-
	4545	+	+	+	-	-	+	+
	8213	+	+	+	-	-	+	+
	344823	+	+	-	+	+	-	+/-
	F38-2E2	+	+	-	+	+	-	-

[0389] As shown in Table 1, since Alexa-647 labeled antibody 512 was found to bind to the TIM-3 expressing cell to which the other unlabeled anti-TIM-3 antibodies were bound, this antibody was suggested to recognize an independent epitope with which antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 did not compete.

[0390] Since it was found that Alexa-647 labeled antibody 644 did not bind or reduced in binding to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 344823 or F38-2E2 was bound, it was indicated that this antibody competed with antibody 344823 or F38-E2E in the recognition of the epitope close to each other.

[0391] Since it was found that Alexa-647 labeled antibody 4545 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 8213 was bound, it was indicated that this antibody competed with antibody 8213 in the recognition of the epitope close to each other.

[0392] Since it was found that Alexa-647 labeled antibody 8213 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 4545 was bound, it was indicated that this antibody competed with antibody 4545 in the recognition of the epitope close to each other.

[0393] Since it was found that APC labeled antibody 344823 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 644 or F38-2E2 was bound, it was indicated that this antibody competed with antibody 644 or F38-E2E antibody in the recognition of the epitope close to each other.

[0394] Since it was found that APC labeled Antibody F38-2E2 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 644 or 344823 was bound, it was indicated that this antibody competed with antibody 644 or antibody 344823 in the recognition of the epitope close to each other.

[Example 20]

(Molecular cloning of mouse TIM-3 cDNA)

[0395] The cDNA of mouse TIM-3 was prepared in the same manner as described in Example 1. Total RNA was extracted from Balb/c mouse derived bone marrow cells using High Pure RNA isolation Kit (manufactured by Roche). Template cDNA was synthesized using ThermoScript RT-PCR system (manufactured by Invitrogen). For primers, mTim-3 Fw3 (SEQ ID NO: 70) and mTim-3 Re3 (SEQ ID NO: 71) were used. The cDNA was inserted into pGEM-T Easy Vector (manufactured by Promega Corp.), and the sequence was analyzed. The clone having identical nucleotide sequence with the coding region of GenBank accession number AF450241 was selected.

[0396] PCR was carried out using the selected clone as the template and inserting the DNA into NotI site of the pEF6/Myc-HisC vector (manufactured by Invitrogen) (mTim-3/pEF6 Myc_HisC). For PCR primers, mTim-3 Fw4NotI (SEQ ID NO: 72) and mTim-3 Re4NotI (SEQ ID NO: 73) were used.

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[Example 21]

(Construction of hTim-3/pEF6 Myc_HisC)

[0397] The plasmid DNA hTIM-3/pMCs-IG prepared in Example 1 and pEF6 Myc_HisC were both digested with NotI to construct human TIM-3 expressing pEF6 Myc_HisC vector (hTim-3/pEF6 Myc_HisC). DNA sequence analysis confirmed that the vector sequence matched the coding region of the GenBank accession number NM_032782.

[Example 22]

(Cross-reactivity of anti-human TIM-3 antibody to anti-mouse TIM-3 antibody)

[0398] Each of the plasmid DNAs, mTim-3/pEF6 Myc_HisC and hTim-3/pEF6 Myc_HisC prepared in Example 20 and Example 21, and an empty vector control was introduced into HEK293F cell in the same manner as Example 4. Two days later, in the same manner as Example 15, the binding activity of Alexa-647 labeled antibodies (antibody 512, antibody 644, antibody 4545 and antibody 8213), commercially available APC labeled anti-human TIM-3 antibodies (antibody 344823 and antibody F38-2E2) and commercially available PE labeled anti-mouse TIM-3 antibody (RMT3-23 antibody, manufactured by BioLegend Inc.) to human TIM-3 and mouse TIM-3 was evaluated using flow cytometry analysis.

[0399] As a result, antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 exclusively interacted with human TIM-3 expressing 293F cells. However, RMT3-23 antibody interacted exclusively with mouse TIM-3 expressing 293F cells. Therefore, it was found that antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 did not cross-react with mouse TIM-3.

[Example 23]

(Construction of expression system for TIM-3 chimeric protein with IgV domain substituted for mouse TIM-3)

[0400] The objective sequence was amplified by the PCR using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mIgV_vecR1 primer (SEQ ID NO: 74) and hTIM3+mIgV_vecF1 primer (SEQ ID NO: 75), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The objective sequence was amplified by PCR using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mIgV_insF1 primer (SEQ ID NO: 76) and hTIM3+mIgV_insR1 primer (SEQ ID NO: 77), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0401] The two PCR products obtained were ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence from the transformant was analyzed and it was found that the PCR products had the objective sequences (IgV chimeraTIM-3/pEF6 Myc_HisC, SEQ ID NO: 78).

[Example 24]

(Construction of expression system for TIM-3 chimeric protein with Mucin domain substituted for mouse TIM-3)

[0402] The objective sequence was amplified by PCR using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mMucin_vecR2 primer (SEQ ID NO: 80) and hTIM3+mMucin_vecF2 primer (SEQ ID NO: 81), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The objective sequence was amplified by PCR using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mMuicn_insF2 primer (SEQ ID NO: 82) and hTIM3+mMucin_insR2 primer (SEQ ID NO: 83), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0403] The two PCR products obtained were ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence from the transformant was analyzed and it was found that the PCR products had the objective sequences(Mucin chimeraTIM-3/pEF6 Myc_HisC, SEQ ID NO: 84).

[Example 25]

(Binding assay of anti-TIM-3 antibody to TIM-3 chimeric protein in which IgV domain was substituted with mouse TIM-3 and TIM-3 chimeric protein in which Mucin domain was substituted with mouse TIM-3)

[0404] Each of the plasmid DNAs prepared in Example 23 and Example 24, IgV chimeraTIM-3/pEF6 Myc_HisC, Mucin chimeraTIM-3/pEF6 Myc_HisC, hTim-3/pEF6 Myc_HisC, mTim-3/pEF6 Myc_HisC, and an empty vector control was introduced into HEK293F cell in the same manner as Example 4. Two days later, in the same manner as Example 22,

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the binding activity of anti-TIM-3 antibody was evaluated using flow cytometry.

[0405] As a result, antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 4177, antibody 344823 and antibody F38-2E2 bound to the human TIM-3 expressing cells and Mucin chimeraTIM-3 expressing cells only. However, RMT-23 antibody bound to the mouse TIM-3 expressing cells and the IgV chimeraTIM-3 expressing cells only.

[0406] Therefore, as a result, it was found that all of the anti-human TIM-3 antibodies tested (antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2) and mouse TIM-3 antibody (antibody RMT3-23) recognized the IgV domain.

[Example 26]

(Construction of TIM-3 chimera 22-47/pEF6 Myc_HisC)

[0407] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 22 to position 47 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 22-47") was constructed. By using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, and hTIM3chimera22-47F1 primer (SEQ ID NO: 86) and hTIM3chimera22-47R1 primer (SEQ ID NO: 87), and PCR was carried out using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0408] Using the obtained PCR product as a template, the objective sequence was amplified by PCR using hTIM3chimera22-47F2 primer (SEQ ID NO: 88)and hTIM3chimera22-47R2 primer (SEQ ID NO: 89) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0409] Using the obtained PCR product as a template, the objective sequence was amplified by PCR using hTIM3chimera22-47F3 primer (SEQ ID NO: 90) and hTIM3chimera22-47R3 primer (SEQ ID NO: 91), and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0410] After the obtained PCR product obtained from the third PCR was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that the clone had the objective sequence (TIM-3 chimera 22-47/pEF6 Myc_HisC, SEQ ID NO: 92).

[Example 27]

(Construction of TIM-3 chimera 57-66/pEF6 Myc_HisC)

[0411] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 57 to position 66 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 57-66") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera57-66F primer (SEQ ID NO: 94) and hTIM3chimera57-66R primer (SEQ ID NO: 95) phosphorylated by T4 Polynucleotide Kinase (manufactured by New England Biolabs) and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.) to amplify the objective sequence.

[0412] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to the agarose gel electrophoresis. The DNA was extracted and ligated with LigaFast™ Rapid DNA Ligation System (manufactured by Promega Corp.). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 57-66/pEF6 Myc_HisC, SEQ ID NO: 96).

[Example 28]

(Construction of TIM-3 chimera 67-105/pEF6 Myc_HisC)

[0413] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 67 to position 105 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 67-105") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3chimera67-105F primer (SEQ ID NO: 98) and hTIM3chimera67-105R primer (SEQ ID NO: 99) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.) to amplify the objective sequence..

[0414] PCR was carried out using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, mTIM3chimera67-105F primer (SEQ ID NO: 100) and mTIM3chimera67-105R primer (SEQ ID NO: 101) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The two PCR products were ligated by GENEART seamless cloning and assembly kit (manufactured by Invitorgen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 67-105/pEF6 Myc_HisC, SEQ ID NO: 102).

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[Example 29]

(Construction of TIM-3 chimera 74-81/pEF6 Myc_HisC)

[0415] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 74 to position 81 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 74-81") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3 chimera74-8 1 F primer (SEQ ID NO: 104) and hTIM3chimera74-81R primer (SEQ ID NO: 105) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0416] After the PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 74-81/pEF6 Myc_HisC, SEQ ID NO: 106).

[Example 30]

(Construction of TIM-3 chimera 88-96/pEF6 Myc_HisC)

[0417] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 88 to position 96 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 88-96") was constructed. The PCR was carried out by preparing a reaction solution containing hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera88-96F primer (SEQ ID NO: 108) and hTIM3chimera88-96R primer (SEQ ID NO: 109) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0418] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 88 -96/pEF6 Myc_HisC, SEQ ID NO: 110).

[Example 31]

(Construction of TIM-3 chimera 96-105/pEF6 Myc_HisC)

[0419] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 96 to position 105 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as "TIM-3 chimera 96-105") was constructed. The PCR was carried out by preparing a reaction solution containing hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera96-105F primer (SEQ ID NO: 112) and hTIM3chimera96-105R primer (SEQ ID NO: 113) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0420] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 96 - 105/pEF6 Myc_HisC, SEQ ID NO: 114).

[Example 32]

(Binding assay of anti-TIM-3 antibody to TIM-3 chimeric protein in which part of the IgV domain was substituted with mouse TIM-3)

[0421] Each of various TIM-3 vectors prepared in Examples 26 to 31 and an empty vector as a control were introduced into HEK293F in the same manner as Example 4. The introduced vectors and the expressed TIM-3 chimeric proteins thereof were as follows:

(1) TIM-3 chimera 22-47: TIM-3 chimeric protein in which the amino acid sequence at position 22 to position 47 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(2) TIM-3 chimera 57-66: TIM-3 chimeric protein in which the amino acid sequence at position 57 to position 66 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(3) TIM-3 chimera 67-105: TIM-3 chimeric protein in which the amino acid sequence at position 67 to position 105

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of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(4) TIM-3 chimera 74-81: TIM-3 chimeric protein in which the amino acid sequence at position 74 to position 81 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(5) TIM-3 chimera 88-96: TIM-3 chimeric protein in which the amino acid sequence at position 88 to position 96 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3; and
(6) TIM-3 chimera 96-105: TIM-3 chimeric protein in which the amino acid sequence at position 96 to position 105 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3.

Two days after transfection, in the same manner as Example 22, the binding activity of anti-TIM-3 antibody was evaluated using flow cytometry.

[0422] The results are shown in Table 2. As shown in Table 2, the case where the binding of the antibody to the transfected cell was detected was indicated as "+", the case where the binding of the antibody to the transfected cell was detected but the degree of the binding was reduced was indicated as "+/-"and the case where the binding of the antibody to the trasfected cell was not detected was indicated as "-".

[Table 2]

Vector name	4545	8213	4177	512	644	344823	F38-2E2
TIM-3 chimera 22-47	+	+	+/-	-	+	+	+
TIM-3 chimera 57-66	+	+	+/-	+	-	-	-
TIM-3 chimera 67-105	-	-	+/-	+	+	+	+
TIM-3 chimera 74-81	-	-	+	+	+	+	+
TIM-3 chimera 88-96	+	+	+	+	+	+	+
TIM-3 chimera 96-105	+	+	+	+	+	+	+

[0423] As shown in Table 2, antibody 4545 and antibody 8213 bound to the cells transfected with TIM3-chimera 22-47, TIM3-chimera 57-66, TIM3-chimera 88-96 or TIM3-chimera 96-105 transfected cells. However, these antibodies did not bind to the cell into which TIM-3 chimera 67-105 were introduced.

[0424] The antibody 512 bound to the cells transfected with TIM3-chimera 57-66, TIM3-chimera 88-96, TIM3-chimera 96-105, TIM-3 chimera 67-105 or TIM-3 chimera 74-81. However, antibody 512 did not bind to the cells transfected with TIM-3 chimera 22-47.

[0425] The antibody 644, antibody 344823 and antibody F38-2E2 bound to the cells transfected with TIM3-chimera 22-47, TIM3-chimera 88-96, TIM3-chimera 96-105, TIM-3 chimera 67-105 or TIM-3 chimera 74-81. However, these antibodies did not bind to the cells transfected with to TIM-3 chimera 57-66.

The antibody 4177 bound to the cells transfected with TIM3-chimera 88-96, TIM3-chimera 96-105 or TIM-3 chimera 74-81. Antibody 4177 weakly bound to TIM-3 the cells transfected with chimera 22-47, TIM-3 chimera 57-66 or TIM-3 chimera 67-105.

[0426] Based on the above result and results from Example 16 and Example 19, it was suggested that antibody 4545 and antibody 8213 bound to the amino acid sequence at position 67 to 87 of human TIM-3. Since antibody 4545 and antibody 8213 did not bind to the cells transfected with TIM-3 chimera 74-81, it was suggested that the amino acids required for binding of antibody 4545 and antibody 8213 to human TIM-3 were included in the amino acid at position 74 to 81.

[0427] In addition, it was suggested that antibody 512 bound to the amino acids up to at position 47 of human TIM-3 and that antibody 644, antibody 344823 and antibody F38- 2E2 bound to the amino acid sequence at position 57 to 66 of human TIM-3.

[0428] Furthermore, it was suggested that antibody 4177 bound to the amino acids other than at position 74 to 81 within the amino acids at position 67 to 87 of human TIM-3 to which antibody 4545 and antibody 8213 bind although antibody 512, antibody 644, antibody 344823 and antibody F38-2E2 did not bind.

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[Example 33]

(Preparation of anti-human TIM-3 antibody 8213 humanized antibody)

(1) Design of amino acid sequences of VH and VL of antibody 8213 humanized antibody

[0429] The amino acid sequence of VH of antibody 8213 humanized antibody was designed in the following manner. The amino acid sequences of FRs in VH of human antibody which is suitable for the grafting of the amino acid sequences (SEQ ID NOs: 21 to 23) of CDRs1 to 3 of antibody 8213 were selected as follows.

[0430] Kabat *et al.,* have classified the VH of conventionally known various human antibodies into three subgroups (HSG I to III) based on the homology of their amino acid sequences and reported the consensus sequences for each of the subgroups [*Sequences of Proteins of Immunological Interest,* US Dept. Health and Human Services (1991)]. Therefore, the homology search of the amino acid consensus sequences of FR of VH subgroups I to III of human antibodies with the amino sequence of FR ofVH of antibody 8213 was conducted.

[0431] As a result of the homology search, the homologies of HSGI, HSGII, and HSGIII were 77.0%, 55.2%, and 58.6%, respectively. Therefore, the amino acid sequence of FR of the VH region of antibody 8213 had the highest homology to the subgroup I.

[0432] Based on the above results, the amino acid sequence of CDR of the VH of antibody 8213 (SEQ ID NOs: 21 to 23) was grafted to an appropriate position of the FR amino acid sequence of the consensus sequence of subgroup I of human antibody VH. In this manner, the 8213 antibody HV0 represented by SEQ ID NO: 67, i.e., the amino acid sequence of VH of an anti-human TIM-3 antibody 8213 humanized antibody was designed.

[0433] Next, the amino acid sequence of VL of antibody 8213 humanized antibody was designed in the following manner. The amino acid sequences of FR in VL of human antibody which were suitable for the grafting of the amino acid sequences (SEQ ID NOs: 24 to 26) of CDRs1 to 3 in VL of antibody 8213 were selected as follows.

[0434] Kabat *et al.,* have classified the VL of various known human antibodies into four subgroups (HSG I to IV) based on the homology of their amino acid sequences and reported the consensus sequences for each of the subgroups [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)]. Therefore, the homology search among the FR amino acid consensus sequences of VL subgroup I to IV of human antibodies were searched for sequence homology with FR amino sequence of VL of antibody 8213.

[0435] As a result of the homology search, the homologies of HSGI, HSGII, HSGIII and HSGIV were 76.3%, 61.3%, 61.3%, and 68.8%, respectively. Therefore, the amino acid sequence of FR of the VL region of antibody 8213 had the highest homology to the subgroup I.

[0436] Based on the above results, each of the amino acid sequences of CDRs of the VL of antibody 8213 (SEQ ID NOs: 24 to 26) was grafted into an appropriate position in the amino acid sequence of FR of the consensus sequence of subgroup I of VL of an human antibody. In this manner, the 8213 antibody LV0 represented by SEQ ID NO: 69, i.e., the amino acid sequence of VL of an anti-human TIM-3 antibody 8213 humanized antibody, was designed.

[0437] The amino acid sequence of 8213 antibody HV0 which was VH of antibody 8213 humanized antibody; and the amino acid sequence of 8213 antibody LV0 which was VL of antibody 8213 humanized antibody designed in the above were the sequences in which only the amino acid sequences of CDRs of the mouse monoclonal antibody 8213 were grafted into the amino acid sequence of the selected FR of human antibody.

[0438] However, in general, it is known that a humanized antibody prepared merely by grafting CDRs of a mouse antibody to FRs of a human antibody has a lower binding activity. In order to avoid decreasing of the binding activity, attempts have been made in the preparation of a humanized antibody to raise the lowered binding activity by modifying the amino acid residues which were considered to have influence on the binding activity among the amino acid sequences of FRs of a human antibody which are different from a mouse antibody as well as grafting amino acid sequences of CDRs. Therefore, in Examples, the inventors decided to identify and modify the amino acid residues of FR which were considered to have influence on the binding activity in the following manner.

[0439] First, three-dimensional structure of the above designed antibody V region (hereinafter referred to as " HV0LV0") comprising the amino acid sequence of antibody 8213 HV0 which was VH of the antibody 8213 humanized antibody and the amino acid sequence of antibody 8213 LV0 which was VL of antibody 8213 humanized antibody was constructed using a computer modeling technique. Discovery Studio (manufactured by Accelrys Inc.) was used for preparation of the three-dimensional structure and display of the three-dimensional structure. A computer model of the three-dimensional structure of V region of antibody 8213 was constructed in the same manner.

[0440] Furthermore, amino acid residues which were different from those of antibody 8213 in the amino acid sequence of FRs ofVH and VL of HV0LV0 were selected, prepared an amino acid sequence in which such amino acid residues were substituted with the corresponding amino acid residues of 8213 antibody and then a three-dimensional structure model was constructed in the same manner. The amino acids which were considered to have influence on the binding activity were selected by comparing the three-dimensional structures of the V regions of antibody 8213 and HV0LV0

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and the modified product.

[0441] As a result, as amino acid residues of FR in HV0LV0 which were considered to change the three-dimensional structure of the antigen binding region and have influence on the binding activity, Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 were selected in antibody 8213 HV0; and Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 were selected in antibody 8213 LV0.

[0442] Among the selected amino acid residues, at least one or more amino acid residues was substituted with amino acid residues which were present at the corresponding sites of antibody 8213 and VHs and VLs of the humanized antibody comprising various modifications were constructed.

[0443] Specifically, as for VH, at least one modification was introduced among the amino acid modifications for substituting Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly, or Val at position 113 with Leu in the amino acid sequence represented by SEQ ID NO: 67.

[0444] As for VL, at least one modification was introduced among the amino acid modifications for substituting Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp in the amino acid sequence represented by SEQ ID NO: 69.

[0445] As the antibody V region of 8213 antibody-humanized antibody in which at least one amino acid existing in FR in HV0LV0 was modified, HV0LV0, HV0LV2, HV0LV4, HV0LV5, HV0LV6, HV0LV7, HV0LV9, HV3LV0, HV3LV2, HV3LV4, HV3LV5, HV3LV6, HV3LV7, HV3LV9, HV4LV0, HV4LV2, HV4LV4, HV4LV5, HV4LV6, HV4LV7, HV4LV9, HV5LV0, HV5LV2, HV5LV4, HV5LV5, HV5LV6, HV5LV7, HV5LV9, HV6LV0, HV6LV2, HV6LV4, HV6LV5, HV6LV6, HV6LV7, HV6LV9, HV7LV0, HV7LV2, HV7LV4, HV7LV5, HV7LV6, HV7LV7, HV7LV9, HV8LV0, HV8LV2, HV8LV4, HV8LV5, HV8LV6, HV8LV7, HV8LV9, HV10LV0, HV10LV2, HV10LV4, HV10LV5, HV10LV6, HV10LV7, HV10LV9, HV12LV0, HV12LV2, HV12LV4, HV12LV5, HV12LV6, HV12LV7, and HV12LV9 were designed.

[0446] The amino acid sequences of each of the H chain variable regions, HV3, HV4, HV5, HV6, HV7, HV8, HV10 and HV12; and the amino acid sequence of the L chain variable region, LV2, LV4, LV5, LV6, LV7 and LV9 were shown in Fig. 1 and Fig. 2, respectively.

(2) Preparation of 8213 antibody-humanized antibody

[0447] DNA encoding the amino acid sequence of the variable region of 8213 antibody-humanized antibody was designed using codons used in the DNAs (SEQ ID NO: 27 and 29) encoding the amino acid sequence of VH of 8213 antibody and VL of 8213 antibody, respectively. When the amino acid modification was introduced, DNA was designed using a codon which was used in a mammal cell with a high frequency.

[0448] Using these DNA sequences, vectors for expressing an antibody were constructed and humanized antibodies were expressed.

[0449] While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

This application is based on U.S. provisional application No. 61/353,836, filed on June 11, 2010, the entire contents of which are incorporated hereinto by reference. All references cited herein are incorporated in their entirety.

Industrial Applicability

[0450] The persent invention can provide a monoclonal antibody or an antibody fragment thereof which binds to the amino acid sequence or the three-dimensional structure thereof of the extracellular region of human TIM-3 and has ADCC activity; a hybridoma which produces the antibody; a DNA which encods the antibody; a vector which comprises the DNA; a transformant which is obtained by transofrming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof as an active ingredient.

Free text of Sequence Listing

[0451]

SEQ ID NO: 1: The amino acid sequence of Human 4545 H chain CDR1

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SEQ ID NO: 2: The amino acid sequence of Human 4545 H chain CDR2
SEQ ID NO: 3: The amino acid sequence of Human 4545 H chain CDR3
SEQ ID NO: 4: The amino acid sequence of Human 4545 L chain CDR1
SEQ ID NO: 5: The amino acid sequence of Human 4545 L chain CDR2
SEQ ID NO: 6: The amino acid sequence of Human 4545 L chain CDR3
SEQ ID NO: 11: The amino acid sequence of Human 4177 H chain CDR1
SEQ ID NO: 12: The amino acid sequence of Human 4177 H chain CDR2
SEQ ID NO: 13: The amino acid sequence of Human 4177 H chain CDR3
SEQ ID NO: 14: The amino acid sequence of Human 4177 L chain CDR1
SEQ ID NO: 15: The amino acid sequence of Human 4177 L chain CDR2
SEQ ID NO: 16: The amino acid sequence of Human 4177 L chain CDR3
SEQ ID NO: 21: The amino acid sequence of Mouse 8213 H chain CDR1
SEQ ID NO: 22: The amino acid sequence of Mouse 8213 H chain CDR2
SEQ ID NO: 23: The amino acid sequence of Mouse 8213 H chain CDR3
SEQ ID NO: 24: The amino acid sequence of Mouse 8213 L chain CDR1
SEQ ID NO: 25: The amino acid sequence of Mouse 8213 L chain CDR2
SEQ ID NO: 26: The amino acid sequence of Mouse 8213 L chain CDR3
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SEQ ID NO: 32: The nucleotide sequence of primer TIM-3 Re2
SEQ ID NO: 33: The nucleotide sequence of primer pMCs-Fw
SEQ ID NO: 34: The nucleotide sequence of primer TIM3ED-FcReXba
SEQ ID NO: 35: The nucleotide sequence of primer T7
SEQ ID NO: 36: The nucleotide sequence of primer hTIM-3 Fw1
SEQ ID NO: 37: The nucleotide sequence of insert hTIM-3 ECD Fc fusion
SEQ ID NO: 38: The amino acid sequence of insert hTIM-3 ECD Fc fusion
SEQ ID NO: 39: The nucleotide sequence of primer TIM3ED-FLAG4aa
SEQ ID NO: 40: The nucleotide sequence of primer C-FLAG-NotR2
SEQ ID NO: 41: The nucleotide sequence of primer BGH-R
SEQ ID NO: 42: The nucleotide sequence of insert hTIM-3 ECD
SEQ ID NO: 43: The amino acid sequence of insert hTIM-3 ECD
SEQ ID NO: 44: The nucleotide sequence of primer hh-6
SEQ ID NO: 45: The nucleotide sequence of primer hh-3
SEQ ID NO: 46: The nucleotide sequence of primer hk-2
SEQ ID NO: 47: The nucleotide sequence of primer hk-6
SEQ ID NO: 48: The nucleotide sequence of primer mH_Rv1
SEQ ID NO: 49: The nucleotide sequence of primer mH_Rv2
SEQ ID NO: 50: The nucleotide sequence of primer mK_Rv1
SEQ ID NO: 51: The nucleotide sequence of primer mK_Rv2
SEQ ID NO: 66: The nucleotide sequence of 8213 antibody HV0 variable region
SEQ ID NO: 67: The amino acid sequence of 8213 antibody HV0 variable region
SEQ ID NO: 68: The nucleotide sequence of 8213 antibody LV0 variable region
SEQ ID NO: 69: The amino acid sequence of 8213 antibody LV0 variable region
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SEQ ID NO: 71: The nucleotide sequence of primer mTim-3 Re3
SEQ ID NO: 72: The nucleotide sequence of primer mTim-3 Fw4NotI
SEQ ID NO: 73: The nucleotide sequence of primer mTim-3 Re4NotI
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SEQ ID NO: 75: The nucleotide sequence of primer hTIM3+mIgV_vecF1
SEQ ID NO: 76: The nucleotide sequence of primer hTIM3+mIgV_insF1
SEQ ID NO: 77: The nucleotide sequence of primer hTIM3+mIgV_insR1
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SEQ ID NO: 79: The amino acid sequence of insert IgV chimeraTIM-3/pEF6 Myc_HisC
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SEQ ID NO: 84: The nucleotide sequence of insert Mucin chimeraTIM-3/pEF6 Myc_HisC
SEQ ID NO: 85: The amino acid sequence of insert Mucin chimeraTIM-3/pEF6 Myc_HisC

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SEQ ID NO: 86: The nucleotide sequence of primer hTIM3chimera22-47F1
SEQ ID NO: 87: The nucleotide sequence of primer hTIM3chimera22-47R1
SEQ ID NO: 88: The nucleotide sequence of primer hTIM3chimera22-47F2
SEQ ID NO: 89: The nucleotide sequence of primer hTIM3chimera22-47R2
SEQ ID NO: 90: The nucleotide sequence of primer hTIM3chimera22-47F3
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SEQ ID NO: 93: The amino acid sequence of insert TIM-3 chimera 22-47/pEF6 Myc_HisC
SEQ ID NO: 94: The nucleotide sequence of primer hTIM3chimera57-66F
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SEQ ID NO: 97: The amino acid sequence of insert TIM-3 chimera 57-66/pEF6 Myc_HisC
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SEQ ID NO: 99: The nucleotide sequence of primer hTIM3chimera67-105R
SEQ ID NO: 100: The nucleotide sequence of primer mTIM3chimera67-105F
SEQ ID NO: 101: The nucleotide sequence of primer mTIM3chimera67-105R
SEQ ID NO: 102: The nucleotide sequence of insert TIM-3 chimera 67-105/pEF6 Myc_HisC
SEQ ID NO: 103: The amino acid sequence of insert TIM-3 chimera 67-105/pEF6 Myc_HisC
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SEQ ID NO: 105: The nucleotide sequence of primer hTIM3chimera74-81R
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SEQ ID NO: 107: The amino acid sequence of insert TIM-3 chimera 74-81/pEF6 Myc_HisC
SEQ ID NO: 108: The nucleotide sequence of primer hTIM3chimera88-96F
SEQ ID NO: 109: The nucleotide sequence of primer hTIM3chimera88-96R
SEQ ID NO: 110: The nucleotide sequence of insert TIM-3 chimera 88-96/pEF6 Myc_HisC
SEQ ID NO: 111: The amino acid sequence of insert TIM-3 chimera 88-96/pEF6 Myc_HisC
SEQ ID NO: 112: The nucleotide sequence of primer hTIM3chimera96-105F
SEQ ID NO: 113: The nucleotide sequence of primer hTIM3chimera96-105R
SEQ ID NO: 114: The nucleotide sequence of insert TIM-3 chimera 96-105/pEF6 Myc_HisC
SEQ ID NO: 115: The amino acid sequence of insert TIM-3 chimera 96-105/pEF6 Myc_HisC

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Kyushu University, National University Corporation

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Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
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Asp Gly Glu Tyr Phe Asp Met Leu Thr Gly Phe Asp Tyr
1 5 10

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<210> 14
<211> 11
<212> PRT
<213> Artificial

<220>
<223> Human4177 L-chain CDR1

<400> 14

Arg Ala Ser Gln Gly Ile Ser Ser Trp Leu Ala
1 5 10

<210> 15
<211> 7
<212> PRT
<213> Artificial

<220>
<223> Human4177 L-chain CDR2

<400> 15

Ala Ala Ser Ser Leu Gln Ser
1 5

<210> 16
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Human4177 L-chain CDR3

<400> 16

Gln Gln Tyr Asn Ser Tyr Pro Arg Thr
1 5

<210> 17
<211> 420
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(420)

<400> 17
atg aaa cat ctg tgg ttc ttc ctt ctc ctg gtg gca gct ccc aga tgg 48
Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
1 5 10 15

gtc ctg tcc cag gtg cag ttg cag gag tcg ggc cca gga ctg gtg aag 96
Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys
20 25 30

cct tcg gag acc ctg tcc ctc acc tgc act gtc tct ggt ggc tcc atc 144

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```
Pro Ser Glu Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile
        35                  40                  45

agt agt tac tac tgg agc tgg atc cgg cag ccc cca ggg aag gga ctg      192
Ser Ser Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu
    50                  55                  60

gag tgg att ggg tat atc ttt cac agt ggg agc acc aac tac aac ccc      240
Glu Trp Ile Gly Tyr Ile Phe His Ser Gly Ser Thr Asn Tyr Asn Pro
65                  70                  75                  80

tcc ctc aag agt cga gtc acc ata tca gta gac acg tcc aag aac cag      288
Ser Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln
                85                  90                  95

ttc tcc ctg aag ctg agc tct gtg acc gct gcg gac acg gcc gtg tat      336
Phe Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr
            100                 105                 110

tac tgt gcg aga gat ggg gag tat ttc gat atg ttg act ggt ttt gac      384
Tyr Cys Ala Arg Asp Gly Glu Tyr Phe Asp Met Leu Thr Gly Phe Asp
        115                 120                 125

tac tgg ggc cag gga acc ctg gtc acc gtc tcc tca                      420
Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135                 140
```

<210> 18
<211> 140
<212> PRT
<213> Homo sapiens

<400> 18

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Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
1               5                   10                  15

Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys
            20                  25                  30

Pro Ser Glu Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile
        35                  40                  45

Ser Ser Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu
    50                  55                  60

Glu Trp Ile Gly Tyr Ile Phe His Ser Gly Ser Thr Asn Tyr Asn Pro
65                  70                  75                  80

Ser Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln
                85                  90                  95

Phe Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr
            100                 105                 110
```


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```
Tyr Cys Ala Arg Asp Gly Glu Tyr Phe Asp Met Leu Thr Gly Phe Asp
        115                 120                 125


Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135                 140


<210>  19
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<212>  DNA
<213>  Homo sapiens


<220>
<221>  CDS
<222>  (1)..(387)

<400>  19
atg gac atg agg gtc ctc gct cag ctc ctg ggg ctc ctg ctg ctc tgt       48
Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15

ttc cca ggt gcc aga tgt gac atc cag atg acc cag tct cca tcc tca       96
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgt cgg gcg agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggt att agc agc tgg tta gcc tgg tat cag cag aaa cca gag aaa      192
Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

gcc cct aag tcc ctg atc tat gct gca tcc agt ttg caa agt ggg gtc      240
Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgc caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

tat aat agt tac cct cgg acg ttc ggc caa ggg acc aag gtg gaa atc      384
Tyr Asn Ser Tyr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
        115                 120                 125

aaa                                                                  387
Lys


<210>  20
<211>  129
<212>  PRT
<213>  Homo sapiens

<400>  20
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Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1 5 10 15

Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
20 25 30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
35 40 45

Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
50 55 60

Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65 70 75 80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
85 90 95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
100 105 110

Tyr Asn Ser Tyr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
115 120 125

Lys

<210> 21
<211> 5
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 H-chain CDR1

<400> 21

Ser Tyr Trp Met His
1 5

<210> 22
<211> 17
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 H-chain CDR2

<400> 22

Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe Lys
1 5 10 15

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Thr

<210> 23
<211> 8
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 H-chain CDR3

<400> 23

Gly Tyr Tyr Leu Tyr Phe Asp Tyr
1 5

<210> 24
<211> 11
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR1

<400> 24

His Ala Ser Gln Gly Ile Arg Ile Asn Ile Gly
1 5 10

<210> 25
<211> 7
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR2

<400> 25

His Gly Thr Asn Leu Glu Asp
1 5

<210> 26
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR3

<400> 26

Val Gln Tyr Gly Gln Phe Pro Trp Thr
1 5

<210> 27
<211> 408

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<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(408)

<400> 27

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atg gga tgg agc tat atc atc ctc ttt ttg gta gca aca gcg aca gat       48
Met Gly Trp Ser Tyr Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Asp
1               5                   10                  15

gtc cac tcc cag gtc caa ctg cag cag cct ggg gct gaa ctg gtg aag       96
Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys
            20                  25                  30

cct ggg gct tca gtg aag ctg tcc tgc aag gct tct ggc tac acc ttc      144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc agc tac tgg atg cac tgg gtg aag cag agg cct gga caa ggc ctt      192
Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg att gga gag att aat cct agc aac ggt cgt act aac tac aat      240
Glu Trp Ile Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
65                  70                  75                  80

gag aag ttc aag acc aag gcc aca ctg act gta gac aaa tcc tcc agc      288
Glu Lys Phe Lys Thr Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
                85                  90                  95

aca gcc tac atg caa ctc agc agc ctg aca tct gag gac tct gcg gtc      336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

tat tac tgt gcg ggg ggt tac tac ctc tac ttt gac tac tgg ggc caa      384
Tyr Tyr Cys Ala Gly Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln
        115                 120                 125

ggc acc act ctc aca gtc tcc tca                                     408
Gly Thr Thr Leu Thr Val Ser Ser
    130                 135
```

<210> 28
<211> 136
<212> PRT
<213> Mus musculus

<400> 28

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Met Gly Trp Ser Tyr Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Asp
1               5                   10                  15

Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45
```


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Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
65 70 75 80

Glu Lys Phe Lys Thr Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Ala Gly Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln
115 120 125

Gly Thr Thr Leu Thr Val Ser Ser
130 135

<210> 29
<211> 387
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(387)

<400> 29

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atg gac atg atg gtc ctt gct cag ttt ctt gca ttc ttg ttg ctt tgg       48
Met Asp Met Met Val Leu Ala Gln Phe Leu Ala Phe Leu Leu Leu Trp
1               5                   10                  15

ttt cca ggt gca aca tgt gac atc ctg atg acc caa tct cca tcc tcc       96
Phe Pro Gly Ala Thr Cys Asp Ile Leu Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

atg tct gta tct ctg gga gac aca gtc aac atc act tgc cat gca agt      144
Met Ser Val Ser Leu Gly Asp Thr Val Asn Ile Thr Cys His Ala Ser
        35                  40                  45

cag ggc att agg att aat ata ggg tgg ttg cag cag aag cca ggg aaa      192
Gln Gly Ile Arg Ile Asn Ile Gly Trp Leu Gln Gln Lys Pro Gly Lys
    50                  55                  60

tca ttt aag ggc ctg atc tat cat gga acc aac ttg gaa gat gga gtt      240
Ser Phe Lys Gly Leu Ile Tyr His Gly Thr Asn Leu Glu Asp Gly Val
65                  70                  75                  80

cca tca agg ttc agt ggc agt gga tct gga cca gat tat tct ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Pro Asp Tyr Ser Leu Thr
                85                  90                  95

atc agc agc ctg gaa tct gaa gat ttt gca gac tat tac tgt gta cag      336
Ile Ser Ser Leu Glu Ser Glu Asp Phe Ala Asp Tyr Tyr Cys Val Gln
```


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100 105 110

tat ggt cag ttt ccg tgg acg ttc ggt gga ggc acc aag ctg gaa atg 384
Tyr Gly Gln Phe Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Met
115 120 125

aaa 387
Lys

<210> 30
<211> 129
<212> PRT
<213> Mus musculus

<400> 30

Met Asp Met Met Val Leu Ala Gln Phe Leu Ala Phe Leu Leu Leu Trp
1 5 10 15

Phe Pro Gly Ala Thr Cys Asp Ile Leu Met Thr Gln Ser Pro Ser Ser
20 25 30

Met Ser Val Ser Leu Gly Asp Thr Val Asn Ile Thr Cys His Ala Ser
35 40 45

Gln Gly Ile Arg Ile Asn Ile Gly Trp Leu Gln Gln Lys Pro Gly Lys
50 55 60

Ser Phe Lys Gly Leu Ile Tyr His Gly Thr Asn Leu Glu Asp Gly Val
65 70 75 80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Pro Asp Tyr Ser Leu Thr
85 90 95

Ile Ser Ser Leu Glu Ser Glu Asp Phe Ala Asp Tyr Tyr Cys Val Gln
100 105 110

Tyr Gly Gln Phe Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Met
115 120 125

Lys

<210> 31
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer TIM-3 Fw2

<400> 31

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gccaccatgt tttcacatct tccctt 26

<210> 32
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer TIM-3 Re2

<400> 32
ctatggcatt gcaaagcgac 20

<210> 33
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Primer pMCs-Fw

<400> 33
tcaaagtaga cggcatcgca g 21

<210> 34
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer TIM3ED-FcReXba

<400> 34
ttttctagat ctgatggttg ctccaga 27

<210> 35
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer T7

<400> 35
taatacgact cactataggg 20

<210> 36
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM-3 Fw1

<400> 36
actctggagc aaccatca 18

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<210> 37
<211> 1333
<212> DNA
<213> Artificial

<220>
<223> Insert hTIM-3 ECD Fc fusion

<220>
<221> CDS
<222> (11)..(1333)

<400> 37

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gattgccacc atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg        49
           Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu
           1               5                   10

ctg ctg cta cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag       97
Leu Leu Leu Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu
    15                  20                  25

gtc ggt cag aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca      145
Val Gly Gln Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro
30                  35                  40                  45

ggg aac ctc gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt      193
Gly Asn Leu Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe
                50                  55                  60

gaa tgt ggc aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat      241
Glu Cys Gly Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr
            65                  70                  75

tgg aca tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg      289
Trp Thr Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val
        80                  85                  90

tcc ctg acc ata gag aat gtg act cta gca gac agt ggg atc tac tgc      337
Ser Leu Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys
    95                  100                 105

tgc cgg atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg      385
Cys Arg Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu
110                 115                 120                 125

aag ttg gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag      433
Lys Leu Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln
                130                 135                 140

aga gac ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat      481
Arg Asp Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His
            145                 150                 155

ggc cca gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta      529
Gly Pro Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu
        160                 165                 170

aca caa ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc      577
Thr Gln Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala
    175                 180                 185

aat gac tta cgg gac tct gga gca acc atc aga tct aga gca gac tac      625
```


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```
Asn Asp Leu Arg Asp Ser Gly Ala Thr Ile Arg Ser Arg Ala Asp Tyr
190                 195                 200                 205

aag gac gac gat gac aag act agt gac aaa act cac aca tgc cca ccg      673
Lys Asp Asp Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro
                210                 215                 220

tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc      721
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
            225                 230                 235

cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca      769
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
        240                 245                 250

tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac      817
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
    255                 260                 265

tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg      865
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
270                 275                 280                 285

gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc      913
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
                290                 295                 300

ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc      961
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
            305                 310                 315

aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa     1009
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
        320                 325                 330

ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gag     1057
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu
    335                 340                 345

gag atg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc     1105
Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
350                 355                 360                 365

tat ccc agc gac atc gcc gcg gag tgg gag agc aat ggg cag ccg gag     1153
Tyr Pro Ser Asp Ile Ala Ala Glu Trp Glu Ser Asn Gly Gln Pro Glu
                370                 375                 380

aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc     1201
Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
            385                 390                 395

ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg     1249
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
        400                 405                 410

aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac     1297
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
    415                 420                 425

acg cag aag agc ctc tcc ctg tct ccg ggt aaa tga                    1333
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
430                 435                 440
```


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<210> 38
<211> 440
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 38

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ser Arg Ala Asp Tyr Lys Asp Asp
        195                 200                 205
```


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```
Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
    210                 215                 220

Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
225                 230                 235                 240

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                245                 250                 255

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
            260                 265                 270

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
        275                 280                 285

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
    290                 295                 300

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
305                 310                 315                 320

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                325                 330                 335

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr
            340                 345                 350

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
        355                 360                 365

Asp Ile Ala Ala Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
    370                 375                 380

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
385                 390                 395                 400

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
                405                 410                 415

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
            420                 425                 430

Ser Leu Ser Leu Ser Pro Gly Lys
        435                 440
```

<210> 39
<211> 30
<212> DNA

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<213> Artificial

<220>
<223> Primer TIM3ED-FLAG4aa

<400> 39
gtccttgtag tctctgatgg ttgctccaga 30

<210> 40
<211> 39
<212> DNA
<213> Artificial

<220>
<223> Primer C-FLAG-NotR2

<400> 40
aaaagcggcc gctcacttgt cgtcatcgtc cttgtagtc 39

<210> 41
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer BGH-R

<400> 41
tagaaggcac agtcgagg 18

<210> 42
<211> 645
<212> DNA
<213> Artificial

<220>
<223> Insert hTIM-3 ECD

<220>
<221> CDS
<222> (19)..(645)

<400> 42
gggaattcga ttgccacc atg ttt tca cat ctt ccc ttt gac tgt gtc ctg 51
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu
1 5 10

ctg ctg ctg ctg cta cta ctt aca agg tcc tca gaa gtg gaa tac aga 99
Leu Leu Leu Leu Leu Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg
15 20 25

gcg gag gtc ggt cag aat gcc tat ctg ccc tgc ttc tac acc cca gcc 147
Ala Glu Val Gly Gln Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala
30 35 40

gcc cca ggg aac ctc gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct 195
Ala Pro Gly Asn Leu Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro
45 50 55

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```
gtg ttt gaa tgt ggc aac gtg gtg ctc agg act gat gaa agg gat gtg      243
Val Phe Glu Cys Gly Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val
60                  65                  70                  75

aat tat tgg aca tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga      291
Asn Tyr Trp Thr Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly
                80                  85                  90

gat gtg tcc ctg acc ata gag aat gtg act cta gca gac agt ggg atc      339
Asp Val Ser Leu Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile
            95                  100                 105

tac tgc tgc cgg atc caa atc cca ggc ata atg aat gat gaa aaa ttt      387
Tyr Cys Cys Arg Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe
        110                 115                 120

aac ctg aag ttg gtc atc aaa cca gcc aag gtc acc cct gca ccg act      435
Asn Leu Lys Leu Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr
    125                 130                 135

cgg cag aga gac ttc act gca gcc ttt cca agg atg ctt acc acc agg      483
Arg Gln Arg Asp Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg
140                 145                 150                 155

gga cat ggc cca gca gag aca cag aca ctg ggg agc ctc cct gat ata      531
Gly His Gly Pro Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile
                160                 165                 170

aat cta aca caa ata tcc aca ttg gcc aat gag tta cgg gac tct aga      579
Asn Leu Thr Gln Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg
            175                 180                 185

ttg gcc aat gac tta cgg gac tct gga gca acc atc aga gac tac aag      627
Leu Ala Asn Asp Leu Arg Asp Ser Gly Ala Thr Ile Arg Asp Tyr Lys
        190                 195                 200

gac gat gac gac aag tga                                             645
Asp Asp Asp Asp Lys
    205
```

<210> 43
<211> 208
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 43

```
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45
```


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Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
165 170 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
180 185 190

Arg Asp Ser Gly Ala Thr Ile Arg Asp Tyr Lys Asp Asp Asp Asp Lys
195 200 205

<210> 44
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer hh-6

<400> 44
ggtccgggag atcatgaggg tgtcctt 27

<210> 45
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hh-3

<400> 45
gtgcacgccg ctggtcaggg cgcctg 26

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<210> 46
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hk-2

<400> 46
gttgaagctc tttgtgacgg gcgagc 26

<210> 47
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hk-6

<400> 47
tggcgggaag atgaagacag atggtg 26

<210> 48
<211> 32
<212> DNA
<213> Artificial

<220>
<223> Primer mH_Rv1

<400> 48
attttgtcga cckyggtsyt gctggcyggg tg 32

<210> 49
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mH_Rv2

<400> 49
gcacacyrct ggacagggat ccagagttcc 30

<210> 50
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mK_Rv1

<400> 50
ttgaagctct tgacaatggg tgaagttgat 30

<210> 51
<211> 30

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<212> DNA
<213> Artificial

<220>
<223> Primer mK_Rv2

<400> 51
gtaggtgctg tctttgctgt cctgatcagt 30

<210> 52
<211> 906
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(906)

<400> 52
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc 384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc 432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca 480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata 528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile

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```
                  165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
```

<210> 53
<211> 301
<212> PRT
<213> Homo sapiens

<400> 53

```
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
```


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85 90 95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
165 170 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
180 185 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
195 200 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
210 215 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225 230 235 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
245 250 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
260 265 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
275 280 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 54
<211> 426
<212> DNA
<213> Homo sapiens

<220>
<221> CDS

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<222> (1)..(426)

<400> 54

```
atg gag ttt ggg ctg agc tgg gtt ttc ctc gtt gct ctt tta aga ggt       48
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
1               5                   10                  15

gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc gtg gtc cag       96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
            20                  25                  30

cct ggg agg tcc ctg aga ctc tcc tgt gca gcg tct gga ttc acc ttc      144
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

aat agc tat ggc atg cac tgg gtc cgc cag gct cca ggc aag ggg ctg      192
Asn Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

gag tgg gtg gca gtt ata tgg tat gat gga agt aat aaa tac tat gga      240
Glu Trp Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Gly
65                  70                  75                  80

gac tcc gtg aag ggc cga ttc acc atc tcc aga gac aat tcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
                85                  90                  95

acg ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gct gtg      336
Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg ata tgg ttc ggg gag atg ttt tcc gaa tac ttc cag      384
Tyr Tyr Cys Ala Ile Trp Phe Gly Glu Met Phe Ser Glu Tyr Phe Gln
        115                 120                 125

cac tgg ggc cag ggc acc ctg gtc acc gtc tcc tca gct agc              426
His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 55
<211> 142
<212> PRT
<213> Homo sapiens

<400> 55

```
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
1               5                   10                  15

Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
            20                  25                  30

Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

Asn Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
```


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```
Glu Trp Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Gly
65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
                85                  90                  95

Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Ile Trp Phe Gly Glu Met Phe Ser Glu Tyr Phe Gln
        115                 120                 125

His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 56
<211> 393
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(393)

<400> 56

```
atg gac atg agg gtc ccc gct cag ctc ctg ggg ctt ctg ctg ctc tgg       48
Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp
1               5                   10                  15

ctc cca ggt gcc aga tgt gcc atc cag ttg acc cag tct cca tcc tcc       96
Leu Pro Gly Ala Arg Cys Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgc cgg gca agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggc att agc agt gct tta gcc tgg tat cag cag aaa cca ggg aaa      192
Gln Gly Ile Ser Ser Ala Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

gct cct aag ctc ctg atc tat gat gcc tcc agt ttg gaa agt ggg gtc      240
Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgt caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

ttt aat agt tac cct ctc act ttc ggc gga ggg acc aag gtg gag atc      384
Phe Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
        115                 120                 125
```


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```
aaa cgt acg                                                              393
Lys Arg Thr
    130
```

<210> 57
<211> 131
<212> PRT
<213> Homo sapiens

<400> 57

```
Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp
1               5                   10                  15

Leu Pro Gly Ala Arg Cys Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

Gln Gly Ile Ser Ser Ala Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

Phe Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
        115                 120                 125

Lys Arg Thr
    130
```

<210> 58
<211> 432
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(432)

<400> 58

```
atg gag ttt ggg ctg agc tgg gtt ttc ctt gtt gct att ata aaa ggt       48
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Ile Lys Gly
1               5                   10                  15
```


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```
gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc ttg gtc aag       96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys
            20                  25                  30

cct gga ggg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc      144
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

agt gac tac tac atg agc tgg atc cgc cag gct cca ggg aag ggg ctg      192
Ser Asp Tyr Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

gag tgg gtt tca ttc att agt agt agt ggt agt atc ata tac tac gca      240
Glu Trp Val Ser Phe Ile Ser Ser Ser Gly Ser Ile Ile Tyr Tyr Ala
65                  70                  75                  80

gac tct gtg aag ggc cga ttc acc atc tcc agg gac aac gcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95

tca ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gct gtg      336
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg aga gat ggg tat agc agc agt tgg tat tac tac ggt      384
Tyr Tyr Cys Ala Arg Asp Gly Tyr Ser Ser Ser Trp Tyr Tyr Tyr Gly
        115                 120                 125

atg gac gtc tgg ggc caa ggg acc acg gtc acc gtc tcc tca gct agc      432
Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 59
<211> 144
<212> PRT
<213> Homo sapiens

<400> 59

```
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Ile Lys Gly
1               5                   10                  15

Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys
            20                  25                  30

Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

Ser Asp Tyr Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

Glu Trp Val Ser Phe Ile Ser Ser Ser Gly Ser Ile Ile Tyr Tyr Ala
65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
```


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```
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Asp Gly Tyr Ser Ser Ser Trp Tyr Tyr Tyr Gly
        115                 120                 125

Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 60
<211> 390
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(390)

<400> 60

```
atg gaa acc cca gcg cag ctt ctc ttc ctc ctg cta ctc tgg ctc cca       48
Met Glu Thr Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1               5                   10                  15

gat acc acc gga gaa att gtg ttg acg cag tct cca ggc acc ctg tct       96
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser
            20                  25                  30

ttg tct cca ggg gaa aga gcc acc ctc tcc tgc agg gcc agt cag agt      144
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45

gtt agc agc agc tac tta gcc tgg tac cag cag aaa cct ggc cag gct      192
Val Ser Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala
    50                  55                  60

ccc agg ctc ctc atc tat ggt gca tcc agc agg gcc act ggc atc cca      240
Pro Arg Leu Leu Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Ile Pro
65                  70                  75                  80

gac agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc      288
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                85                  90                  95

agc aga ctg gag cct gaa gat ttt gca gtg tat tac tgt cag cag tat      336
Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr
            100                 105                 110

ggt agc tca ccg ctc act ttc ggc gga ggg acc aag gtg gag atc aaa      384
Gly Ser Ser Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

cgt acg                                                             390
Arg Thr
    130
```

<210> 61
<211> 130

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<212> PRT
<213> Homo sapiens

<400> 61

Met Glu Thr Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1 5 10 15

Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser
20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
35 40 45

Val Ser Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala
50 55 60

Pro Arg Leu Leu Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Ile Pro
65 70 75 80

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
85 90 95

Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr
100 105 110

Gly Ser Ser Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
115 120 125

Arg Thr
130

<210> 62
<211> 414
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(414)

<400> 62
atg gac tgg acc tgg agc atc ctt ttc ttg gtg gca gca gca aca ggt 48
Met Asp Trp Thr Trp Ser Ile Leu Phe Leu Val Ala Ala Ala Thr Gly
1 5 10 15

gcc cac tcc cag gtt cag ctg gtg cag tct gga gct gag gtg aag aag 96
Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

cct ggg gcc tca gtg aag gtc tcc tgc aag gct tct ggt tac acc ttt 144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

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```
acc agc tat ggt atc agc tgg gtg cga cag gcc cct gga caa ggg ctt      192
Thr Ser Tyr Gly Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg atg gga tgg atc agc gct tac aat ggt aac aca aac tat gca      240
Glu Trp Met Gly Trp Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala
65                  70                  75                  80

cag aag ctc cag ggc aga gtc acc atg acc aca gac aca tcc acg agc      288
Gln Lys Leu Gln Gly Arg Val Thr Met Thr Thr Asp Thr Ser Thr Ser
                85                  90                  95

aca gcc tac atg gag ctg agg agc ctg aga tct gac gac acg gcc gtg      336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg aga tgg gat agc agc agc tgg tcc ttt gac tac tgg      384
Tyr Tyr Cys Ala Arg Trp Asp Ser Ser Ser Trp Ser Phe Asp Tyr Trp
        115                 120                 125

ggc cag gga acc ctg gtc acc gtc tcc tca                              414
Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135
```

<210> 63
<211> 138
<212> PRT
<213> Homo sapiens

<400> 63

```
Met Asp Trp Thr Trp Ser Ile Leu Phe Leu Val Ala Ala Ala Thr Gly
1               5                   10                  15

Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Ser Tyr Gly Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Met Gly Trp Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala
65                  70                  75                  80

Gln Lys Leu Gln Gly Arg Val Thr Met Thr Thr Asp Thr Ser Thr Ser
                85                  90                  95

Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Trp Asp Ser Ser Ser Trp Ser Phe Asp Tyr Trp
        115                 120                 125
```


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```
Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135


<210>  64
<211>  390
<212>  DNA
<213>  Homo sapiens


<220>
<221>  CDS
<222>  (1)..(390)

<400>  64
atg gac atg agg gtc ctc gct cag ctc ctg ggg ctc ctg ctg ctc tgt       48
Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15

ttc cca ggt gcc aga tgt gac atc cag atg acc cag tct cca tcc tca       96
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgt cgg gcg agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggt att agc agc tgg tta gcc tgg tat cag cag aaa cca gag aaa      192
Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

gcc cct aag tcc ctg atc tat gct gca tcc agt ttg caa agt ggg gtc      240
Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgc caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

tat aat agt tac ccg tac act ttt ggc cag ggg acc aag ctg gag atc      384
Tyr Asn Ser Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
        115                 120                 125

aaa cgt                                                             390
Lys Arg
    130


<210>  65
<211>  130
<212>  PRT
<213>  Homo sapiens

<400>  65

Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15
```


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```
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

Tyr Asn Ser Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
        115                 120                 125

Lys Arg
    130
```

<210> 66
<211> 351
<212> DNA
<213> Artificial

<220>
<223> HV0 Mouse8213 VH

<220>
<221> CDS
<222> (1)..(351)

<400> 66

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cag gtc caa ctg gtg cag tct ggg gct gaa gtg aag aag cct ggg gct       48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                   10                  15

tca gtg aag gtg tcc tgc aag gct tct ggc tac acc ttc acc agc tac       96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

tgg atg cac tgg gtg cgg cag gcg cct gga caa ggc ctt gag tgg atg      144
Trp Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

gga gag att aat cct agc aac ggt cgt act aac tac aat gag aag ttc      192
Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe
    50                  55                  60
```


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aag acc cgg gtc aca atc act gca gac aca tcc acc agc aca gcc tac 240
Lys Thr Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

atg gaa ctc agc agc ctg cga tct gag gac act gcg gtc tat tac tgt 288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

gcg cgg ggt tac tac ctc tac ttt gac tac tgg ggc caa ggc acc ctg 336
Ala Arg Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

gtc aca gtc tcc tca 351
Val Thr Val Ser Ser
115

<210> 67
<211> 117
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 67

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Trp Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe
50 55 60

Lys Thr Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala 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 Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

Val Thr Val Ser Ser
115

<210> 68
<211> 321
<212> DNA

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<213> Artificial

<220>
<223> LV0 Mouse8213 VL

<220>
<221> CDS
<222> (1)..(321)

<400> 68

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gac atc cag atg acc caa tct cca tcc tcc ttg tct gca tct gtg gga       48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

gac cga gtc acc atc act tgc cat gca agt cag ggc att agg att aat       96
Asp Arg Val Thr Ile Thr Cys His Ala Ser Gln Gly Ile Arg Ile Asn
            20                  25                  30

ata ggg tgg tat cag cag aag cca ggg aaa gca cct aag ctc ctg atc      144
Ile Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

tat cat gga acc aac ttg gaa gat gga gtt cca tca agg ttc agt ggc      192
Tyr His Gly Thr Asn Leu Glu Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

agt gga tct gga aca gat ttc act ctc acc atc agc agc ctg caa cct      240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

gaa gat ttt gca acc tat tac tgt gta cag tat ggt cag ttt ccg tgg      288
Glu Asp Phe Ala Thr Tyr Tyr Cys Val Gln Tyr Gly Gln Phe Pro Trp
                85                  90                  95

acg ttc ggt cag ggc acc aag ctg gaa atc aaa                         321
Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 69
<211> 107
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 69

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys His Ala Ser Gln Gly Ile Arg Ile Asn
            20                  25                  30

Ile Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr His Gly Thr Asn Leu Glu Asp Gly Val Pro Ser Arg Phe Ser Gly
```


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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 Val Gln Tyr Gly Gln Phe Pro Trp
85 90 95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 70
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Fw3

<400> 70
ctacacagag ctgtccttgg at 22

<210> 71
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Re3

<400> 71
tttctcagtg gctgtggtca 20

<210> 72
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Fw4NotI

<400> 72
aattgcggcc gccaccatgt tttcaggtct tac 33

<210> 73
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Re4NotI

<400> 73
aattgcggcc gctcaggatg gctgctggct 30

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<210> 74
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mIgV_vecR1

<400> 74
ggaccttgta agtagtagca 20

<210> 75
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mIgV_vecF1

<400> 75
gccaaggtca cccctgcacc ga 22

<210> 76
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mIgV_insF1

<400> 76
ctacttacaa ggtccttgga agatggttat aaggt 35

<210> 77
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mIgV_insR1

<400> 77
aggggtgacc ttggctgctt tgatgtctaa tttca 35

<210> 78
<211> 909
<212> DNA
<213> Artificial

<220>
<223> IgV chimeraTIM-3/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 78
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48

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```
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc ttg gaa gat ggt tat aag gtt gag gtt ggt aaa   96
Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30

aat gcc tat ctg ccc tgc agt tac act cta cct aca tct ggg aca ctt  144
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

gtg cct atg tgc tgg ggc aag gga ttc tgt cct tgg tca cag tgt acc  192
Val Pro Met Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

aat gag ttg ctc aga act gat gaa aga aat gtg aca tat cag aaa tcc  240
Asn Glu Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

agc aga tac cag cta aag ggc gat ctc aac aaa gga gat gtg tct ctg  288
Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

atc ata aag aat gtg act ctg gat gac cat ggg acc tac tgc tgc agg  336
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Thr Tyr Cys Cys Arg
            100                 105                 110

ata cag ttc cct ggt ctt atg aat gat aaa aaa tta gaa ctg aaa tta  384
Ile Gln Phe Pro Gly Leu Met Asn Asp Lys Lys Leu Glu Leu Lys Leu
        115                 120                 125

gac atc aaa gca gcc aag gtc acc cct gca ccg act cgg cag aga gac  432
Asp Ile Lys Ala Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca  480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa  528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac  576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca  624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att  672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc  720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca  768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255
```


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```
gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  79
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  79

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

Val Pro Met Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

Asn Glu Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Thr Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Phe Pro Gly Leu Met Asn Asp Lys Lys Leu Glu Leu Lys Leu
        115                 120                 125

Asp Ile Lys Ala Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160
```


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Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
165 170 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
180 185 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
195 200 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
210 215 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225 230 235 240

Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
245 250 255

Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
260 265 270

Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
275 280 285

Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 80
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_vecR2

<400> 80
tggtttgatg accaacttca g 21

<210> 81
<211> 23
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_vecF2

<400> 81
ataggcatct acatcggagc agg 23

<210> 82
<211> 35
<212> DNA

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<213> Artificial

<220>
<223> Primer hTIM3+mMucin_insF2

<400> 82
ttggtcatca aaccagccaa ggtcactcca gctca 35

<210> 83
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_insR2

<400> 83
gatgtagatg cctattctga tcgtttctcc agagt 35

<210> 84
<211> 876
<212> DNA
<213> Artificial

<220>
<223> Mucin chimeraTIM-3/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(876)

<400> 84
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

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```
caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc      384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

atc aaa cca gcc aag gtc act cca gct cag act gcc cat ggg gac tct      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Gln Thr Ala His Gly Asp Ser
    130                 135                 140

act aca gct tct cca aga acc cta acc acg gag aga aat ggt tca gag      480
Thr Thr Ala Ser Pro Arg Thr Leu Thr Thr Glu Arg Asn Gly Ser Glu
145                 150                 155                 160

aca cag aca ctg gtg acc ctc cat aat aac aat gga aca aaa att tcc      528
Thr Gln Thr Leu Val Thr Leu His Asn Asn Asn Gly Thr Lys Ile Ser
                165                 170                 175

aca tgg gct gat gaa att aag gac tct gga gaa acg atc aga ata ggc      576
Thr Trp Ala Asp Glu Ile Lys Asp Ser Gly Glu Thr Ile Arg Ile Gly
            180                 185                 190

atc tac atc gga gca ggg atc tgt gct ggg ctg gct ctg gct ctt atc      624
Ile Tyr Ile Gly Ala Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile
        195                 200                 205

ttc ggc gct tta att ttc aaa tgg tat tct cat agc aaa gag aag ata      672
Phe Gly Ala Leu Ile Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile
    210                 215                 220

cag aat tta agc ctc atc tct ttg gcc aac ctc cct ccc tca gga ttg      720
Gln Asn Leu Ser Leu Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu
225                 230                 235                 240

gca aat gca gta gca gag gga att cgc tca gaa gaa aac atc tat acc      768
Ala Asn Ala Val Ala Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr
                245                 250                 255

att gaa gag aac gta tat gaa gtg gag gag ccc aat gag tat tat tgc      816
Ile Glu Glu Asn Val Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys
            260                 265                 270

tat gtc agc agc agg cag caa ccc tca caa cct ttg ggt tgt cgc ttt      864
Tyr Val Ser Ser Arg Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe
        275                 280                 285

gca atg cca tag                                                      876
Ala Met Pro
    290
```

<210> 85
<211> 291
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 85

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15
```


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```
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Gln Thr Ala His Gly Asp Ser
    130                 135                 140

Thr Thr Ala Ser Pro Arg Thr Leu Thr Thr Glu Arg Asn Gly Ser Glu
145                 150                 155                 160

Thr Gln Thr Leu Val Thr Leu His Asn Asn Asn Gly Thr Lys Ile Ser
                165                 170                 175

Thr Trp Ala Asp Glu Ile Lys Asp Ser Gly Glu Thr Ile Arg Ile Gly
            180                 185                 190

Ile Tyr Ile Gly Ala Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile
        195                 200                 205

Phe Gly Ala Leu Ile Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile
    210                 215                 220

Gln Asn Leu Ser Leu Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu
225                 230                 235                 240

Ala Asn Ala Val Ala Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr
                245                 250                 255

Ile Glu Glu Asn Val Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys
            260                 265                 270
```


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Tyr Val Ser Ser Arg Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe
275 280 285

Ala Met Pro
290

<210> 86
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47F1

<400> 86
acatctggga cacttgtgcc cgtctgctgg ggcaa 35

<210> 87
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R1

<400> 87
ataaccatct tccaaggacc ttgtaagtag tagca 35

<210> 88
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47F2

<400> 88
ctgcagttac actctaccta catctgggac acttg 35

<210> 89
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R2

<400> 89
ttaccaacct caaccttata accatcttcc aagga 35

<210> 90
<211> 35
<212> DNA
<213> Artificial

<220>

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<223> Primer hTIM3chimera22-47F3

<400> 90
aaatgcctat ctgccctgca gttacactct accta 35

<210> 91
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R3

<400> 91
ggcagatagg cattttacc aacctcaacc tta 33

<210> 92
<211> 906
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 22-47/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(906)

<400> 92
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc ttg gaa gat ggt tat aag gtt gag gtt ggt aaa 96
Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
20 25 30

aat gcc tat ctg ccc tgc agt tac act cta cct aca tct ggg aca ctt 144
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc 384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

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```
atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca      480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata      528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
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<210> 93
<211> 301
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 93

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30
```


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Page 918

```
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285
```


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Page 919

```
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  94
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<212>  DNA
<213>  Artificial

<220>
<223>  Primer hTIM3chimera57-66F

<400>  94
acagtgtacc aatgaggtgc tcaggactga tgaaa                                  35

<210>  95
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<212>  DNA
<213>  Artificial

<220>
<223>  Primer hTIM3chimera57-66R

<400>  95
gaccaaggac agaatccttt gccccagcag acg                                    33

<210>  96
<211>  906
<212>  DNA
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<220>
<223>  TIM-3 chimera 57-66/pEF6 Myc_HisC


<220>
<221>  CDS
<222>  (1)..(906)

<400>  96
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta       48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag       96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc      144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

gtg ccc gtc tgc tgg ggc aaa gga ttc tgt cct tgg tca cag tgt acc      192
Val Pro Val Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

aat gag gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc      240
Asn Glu Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80
```


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```
aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc      288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc      336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc      384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca      480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata      528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
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<210> 97
<211> 301
<212> PRT
<213> Artificial

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<220>
<223> Synthetic Construct

<400> 97

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

Asn Glu Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240
```


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Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
245 250 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
260 265 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
275 280 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 98
<211> 34
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera67-105F

<400> 98
ctggatgacc atgggatcta ctgctgccgg atcc 34

<210> 99
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera67-105R

<400> 99
atcagttctg agcaacacgt tgccacattc aaa 33

<210> 100
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTIM3chimera67-105F

<400> 100
ttgctcagaa ctgatgaaag 20

<210> 101
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTIM3chimera67-105R

<400> 101

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cccatggtca tccagagtca 20

<210> 102
<211> 909
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 67-105/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 102

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag       96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc      144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc      192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

aac gtg ttg ctc aga act gat gaa aga aat gtg aca tat cag aaa tcc      240
Asn Val Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

agc aga tac cag cta aag ggc gat ctc aac aaa gga gat gtg tct ctg      288
Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

atc ata aag aat gtg act ctg gat gac cat ggg atc tac tgc tgc cgg      336
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Ile Tyr Cys Cys Arg
            100                 105                 110

atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg      384
Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac      432
Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca      480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa      528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac      576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
```


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```
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca      624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att      672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc      720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca      768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  103
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  103

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95
```


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Page 925

```
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Ile Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300

<210>  104
<211>  35
<212>  DNA
<213>  Artificial

<220>
<223>  Primer hTIM3chimera74-81F

<400>  104
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gtgacatatc agaaatcctc cagatactgg ctaaa 35

<210> 105
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera74-81R

<400> 105
tttctgatat gtcacattcc tttcatcagt cctga 35

<210> 106
<211> 909
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 74-81/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 106

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atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta   48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag   96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc  144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc  192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

aac gtg gtg ctc agg act gat gaa agg aat gtg aca tat cag aaa tcc  240
Asn Val Val Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg  288
Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu
                85                  90                  95

acc ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg  336
Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg
            100                 105                 110

atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg  384
Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac  432
Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
```


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Page 927

```
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca      480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa      528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac      576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca      624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att      672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc      720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca      768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  107
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  107

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45
```


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Page 928

```
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu
                85                  90                  95

Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

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tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
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tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
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gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
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225                 230                 235                 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
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Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
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Claims

1. A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (i) to (iii):

   (i) an antibody which comprises an H chain of an antibody which comprises complementary determining regions CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
   (ii) an antibody which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
   (iii) an antibody which comprises an H chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

2. The monoclonal antibody or the antibody fragment thereof according to claim 1, wherein the monoclonal antibody binds to the same epitope to which one antibody selected from the above (i) to (iii) binds.

3. A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (a) and (b):

   (a) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
   (b) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

4. The monoclonal antibody or the antibody fragment thereof according to claim 3, which binds to the same epitope to which one antibody selected from the above (a) or (b) binds.

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5. The monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 4, which is a recombinant antibody.

6. The monoclonal antibody or the antibody fragment thereof according to claim 5, which is a recombinant antibody selected from a human chimeric antibody, a humanized antibody and a human antibody.

7. A monoclonal antibody or an antibody fragment thereof, which is one selected from the following (i) to (iii):

(i) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

8. A monoclonal antibody and an antibody fragment thereof, which is one selected from the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

9. A monoclonal antibody and an antibody fragment thereof which binds to the amino acid sequences at positions 67 to 105, the amino acid sequences at positions 67 to 96 or the amino acid sequences at positions 67 to 87 in the amino acid sequence of IgV domain of human TIM-3 represented by SEQ ID NO: 53.

10. The antibody fragment according to any one of claims 1 to 9, wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv) and a peptide comprising CDR.

11. A DNA which encodes the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

12. A recombinant vector which comprises the DNA according to claim 11.

13. A transformant obtainable by introducing the recombinant vector according to claim 12 into a host cell.

14. A process for producing the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10, which comprises culturing the transformant according to claim 13 in culture to form and accumulate the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10, and recovering the monoclonal antibody or the antibody fragment thereof from the culture.

15. A method for immunologically detecting or measuring a human TIM-3 which comprises using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

16. A reagent for detecting or measuring a human TIM-3 which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

17. A diagnostic agent for a disease relating to a human TIM-3 positive cell which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

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18. A method for diagnosing a disease relating to a human TIM-3 positive cell which comprises detect or measure a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

19. A method for diagnosing a disease relating to a human TIM-3 positive cell, which comprises ditecting or measuring a human TIM-3 using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

20. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10 for the manufacture of a diagnostic agent for a disease relating to a human TIM-3 positive cell.

21. A therapeutic agent for a disease relating to a human TIM-3 positive cell, which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

22. A therapeutic method for a disease relating to a human TIM-3 positive cell, which comprises inducing cell death of a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

23. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10 for the manufacture of a therapeutic agent for a disease relating to a human TIM-3 positive cell.

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Fig. 1

	1	2	3	4	5	6
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8213 antibody HV3				K	I	
8213 antibody HV4				K	I	
8213 antibody HV5				K R	I	
8213 antibody HV6		V				
8213 antibody HV7				K R	I	
8213 antibody HV8			L	K	I	
8213 antibody HV10		V		K R	I	
8213 antibody HV12		V	L	K R	I	

	7	8	9	10	11	
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8213 antibody HV0	NEKFKTRVTI	TADTSTSTAY	MELSSLRSED	TAVYYCARGY	YLYFDYWGQG	TLVTVSS
8213 antibody HV3				G		
8213 antibody HV4	K	K				
8213 antibody HV5	K			G		
8213 antibody HV6	KA	V K		G		
8213 antibody HV7	K	V K		G		
8213 antibody HV8	A L	V		G	L	
8213 antibody HV10	KA L	V K		G		
8213 antibody HV12	KA L	V K		G		

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Fig. 2

	1	2	3	4	5	6
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8213 antibody LV2				L	F	
8213 antibody LV4		L		L	G	
8213 antibody LV5				L	SF G	
8213 antibody LV6		L		L	F G	
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8213 antibody LV9		M V L		L	SF G	

	7	8	9	10	
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

A. CLASSIFICATION OF SUBJECT MATTER
A61P7/00(2006.01)i, *A61K39/00*(2006.01)i, *C07K16/28*(2006.01)i, *C12N1/15*(2006.01)i, *C12N1/19*(2006.01)i, *C12N1/21*(2006.01)i, *C12N5/10*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/08*(2006.01)i
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P7/00, A61K39/00, C07K16/28, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/09, C12P21/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2011
Kokai Jitsuyo Shinan Koho 1971-2011 Toroku Jitsuyo Shinan Koho 1994-2011

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubMed, JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2010-510223 A (THE BRIGHAM AND WOMEN'S HOSPITAL, INC.), 02 April 2010 (02.04.2010), & US 2010/0061992 A1 & EP 2081961 A2 & WO 2008/060617 A2 & CA 2668693 A1	1-17,20,21, 23
Y	JP 2005-526018 A (THE BRIGHAM AND WOMEN'S HOSPITAL, INC., DANA-FARBER CANCER INSTITUTE, INC.), 02 September 2005 (02.09.2005), & US 2004/0005322 A1 & EP 1467759 A2 & WO 2003/063792 A2	1-17,20,21, 23
Y	JP 2007-530560 A (TELOS PHARMACEUTICALS, INC.), 01 November 2007 (01.11.2007), & US 2005/0276756 A1 & EP 1740224 A2 & WO 2005/097211 A2 & KR 10-2007-0037570 A	1-17,20,21, 23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 September, 2011 (06.09.11)	13 September, 2011 (13.09.11)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	IIDA S., et al., Nonfucosylated Therapeutic IgG1 Antibody Can Evade the Inhibitory Effect of Serum Immunoglobulin G on Antibody-Dependent Cellular Cytotoxicity through its High Binding to FcγRIIIa., Clin. Cancer Res., 2006, Vol.12, No.9, p.2879-2887	1-17,20,21, 23
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Form PCT/ISA/210 (continuation of second sheet) (July 2009)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [x] Claims Nos.: 18,19,22
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 18, 19 and 22 pertain to diagnostic methods to be practiced on the human body or methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under the provisions of PCT Rule 39.1(iv), to search.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

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REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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20 July 2011

Dear Sirs

European Patent Application No. 03809145.0-2402
GENMAB A/S
Our Ref: N.94565 GCW/no

Thank you for the Communication under Rule 71(3) EPC dated 31 March 2011. As a preliminary response, I kindly request amendment of the application as follows:

- please replace pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44 at present on file by correspondingly-numbered new pages provided;
- please replace pages 1 to 7 of the sequence listing by the correspondingly-numbered new sequence listing pages provided; and
- please replace pages 110 and 111 at present on file by the correspondingly-numbered new pages provided.

New pages 2, 3 and 31 have been retyped. I therefore also attach a hand-amended version of original pages 2, 3 and 31, i.e of pages 2, 3 and 31 of the description of the original International Application Publication No. WO 2004/035607, showing how the new pages have been derived. Similarly, I attach a hand-amended version of original pages 1 to 7 of the sequence listing showing how the new sequence listing pages have been derived.

New Pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44

I am filing replacement pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44 in order to conform the description more precisely to the invention now claimed. I wish to avoid any possible difficulties

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and ambiguities in interpretation of the granted claims.

The description which was amended by the Examining Division to derive the description of the Druckexemplar was in fact the description on file on the basis of the response filed 23 February 2007 to the first Examination Report dated 28 April 2006. In that response, replacement pages 2, 3, 3a to 3c, 5, 6, 6a, 8, 32 and 35 were submitted in order to bring the description into conformity with the replacement claims that were being filed at that time. Exact counterparts to some of those claims were therefore included in the description.

However, the claims which have now been allowed and which are incorporated in the Druckexemplar are rather different from the claims submitted in response to the first Examination Report. It is therefore inappropriate to retain in the description the passages which were added on 23 February 2007 in the response to the first Examination Report to provide counterparts to the claims being filed at that time. Indeed, the presence of these passages risks causing confusion about the interpretation of the claims.

In order to derive the new pages of the description which are being filed now, I have returned to the original pages of the description, i.e. to the pages of the description of WO 2004/035607. I have thus retained the original disclosure wherever possible. I have provided an exact counterpart to allowed claim 1 on new page 2 at the beginning of the *"Summary of the Invention"* section. Subsequent pages have been revised to delete references to antibody 11B8 as an antibody of the invention.

Lines 23 to 34 on page 3 have been deleted because this passage is broader in scope than allowed claim 1. I have retained mention of *"the invention"* and not referred instead to *"the disclosure"*. The relevant passages do still relate to the claimed invention. Further, I wish to avoid problems which might arise post-grant concerning the scope of the invention if the term *"the disclosure"* were to be retained. Thus, I wish to avoid inconsistencies between the claims and the description which could render the scope of the claims unclear.

Correction of Sequence Listing

I request correction under Rule 139 EPC of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 13, 19 and 25 of the sequence listing. These corrections fall into two categories:

- First, I request correction SEQ ID NOs: 2, 4, 6, 8, 10 and 12 to remove the leader sequences that were erroneously included.

- Second, I request correction SEQ ID NOs: 13, 19 and 25 to remove the extra N-terminal amino acid which was erroneously included in each case.

For all of the requested corrections, it is immediately evident that an error has occurred and what the

correction should be (Examination Guidelines C-VI-5.4). In that regard, I attach a copy of a Declaration from Dr Tom Vink, Associate Director, Cell & Molecular Sciences, Genmab BV, and a copy of the attachments to which the Declaration refers. Please see Dr Vink's explanations.

Claims 38 and 40

I wish to clarify claims 38 and 40. These two claims as amended in the Druckexemplar refer to a human antibody, composition, expression vector, immunoconjugate or bispecific molecule "*for the use according to claim 37*" or "*for the use according to claim 39*" respectively. However, claims 37 and 39 are not "*use*" claims.

On a literal interpretation, claims 38 and 40 could now be considered as being directed to any human antibody, composition, expression vector, immunoconjugate or bispecific molecule for use in treating or preventing follicular lymphoma, B cell chronic lymphocytic leukaemia or small lymphocytic lymphoma (claim 38) or rheumatoid arthritis (claim 40). I therefore wish to amend the two claims to avoid any such possible implication whilst retaining the correct claim format for second medical use claims.

Our further response will follow in due course enclosing the corresponding translations of the claims into French and German and a debit order authorising deduction of the necessary fees from our deposit account 2805.0038.

Yours faithfully



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Our ref: SK/6930 Your ref: **EP 3 167 888** Wroclaw, September 4th, 2019

Re: **Third Party Observations in respect of EP 3 167 888**

THIRD PARTY OBSERVATIONS

European Patent:	Application Number: EP 16195355.9
	Publication Number: EP 3 167 888
Applicant:	Alexion Pharmaceuticals, Inc.
Title:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Dear Sirs,

We enclose the Third Party Observations in respect of EP 3 167 888 for attention of Examining Division.

Your faithfully

Rafał Witek
Patent Attorney

Enclosures:

1. Third Party Observations in respect of EP 3 167 888
2. EP1558648_Request for correction
3. EP1558648_Original filing (WO 2004/035607 A2)
4. EP1558648_Vink declaration
5. EP2581113_Request for correction
6. EP2581113_Application as filed

4th September, 2019

EP Appl. No. 16 195 355.9 – 1466 (EP 3 167 888)
In the name of Alexion Pharmaceuticals, Inc.

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

I. The amended claims submitted by the Applicant as Main Request contravene Articles 123(2) and 76(1) EPC

(1) In response to the Examining Division's Communication in preparation of oral proceedings, dated May 7, 2018, the Applicant argued in submissions dated August 15, 2018 and July 5, 2019, that the skilled person would have immediately recognized that the Applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he/she would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

(2) The Applicant pointed to Enlarged Board of Appeal decisions **G 3/89** and **G 11/91** to support his allegations. We note that the Applicant's arguments are based on a flawed interpretation of the cited case law.

(3) A correction under Rule 139 EPC is of a strictly declaratory nature. Two requirements arise for a correction under Rule 139 to be allowable, i.e., not to contravene Article 123(2) EPC and/or Article 76(1) EPC. First, the presence of an error must be obvious. Second, it has to be *"immediately evident [for a skilled person] that nothing else would have been intended than what is offered as the correction"* (Rule 139 EPC, second sentence). If there is any doubt, including even slight ambivalence, that nothing else would have been intended than what is offered as the correction (i.e., the correct sequence of eculizumab), a correction cannot be allowed (see also **J 16/13**, points 8 to 10 of the reasons).

(4) The Proprietor has not met these requirements as they have not shown that it would be immediately evident that the leader sequence of SEQ ID NOs: 3 and 4 would precisely consist of residues 1-22 of SEQ ID NOs: 3 and 4 and nothing else.

(5) The Proprietor alleges in this regard (par. bridging p. 2/3 of the submission dated August 15, 2018) that the skilled person would know from his common general knowledge that the *"framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat et al."* (Exhibit E filed with the Andrien Declaration on July 10, 2017). Notably, the table

of contents (Exhibit C filed with the Andrien Declaration on July 10, 2017) makes clear that the Applicant chose to submit only a fraction of the human kappa light chain sequences disclosed in Kabat *et al.* (according to the table of contents, human kappa light chain sequences are disclosed on pages 103 to 130 of Kabat *et al.* out of which only pages 103 to 108 have been submitted). The sequence variability among human kappa light chains must therefore be assumed to be considerably greater than what is derivable from the documents submitted by the Applicant.

(6) However, even within the limited selection presented by the Applicant, Asp lle Gln Met are at best commonly the first residues. When assessing the allowability of an amendment under Rule 139 EPC, the question is not whether human kappa light chains commonly start with any particular sequence, but whether they must start with a certain sequence and whether this would have been obvious to the skilled person. Otherwise, the skilled person would be left with uncertainty, at which specific residue of SEQ IDs NO: 3 and 4 the correct mature sequence (excluding the leader peptide) would begin. This is clearly the case here, which will we explained in more detail below.

(7) Specifically, one question is how many residues the skilled person would have subtracted from the sequence disclosed in the patent, if recognizing that said sequences comprise a leader peptide? Further, how would the skilled person arrive at one and only one possible sequence to deduct from SEQ ID NOs: 3 and 4? The Proprietor states that *"the last two residues [of the leader peptides] are predominantly Arg Cys"* (par. bridging p. 2/3 of the submission). Again, as the Examining Division will be aware, *"predominantly"* does not meet the standard required for a correction under Rule 139 EPC. Quite to the contrary, the fact that the Applicant uses the term "predominantly" already shows that there are alternatives to the correction that would be apparent to the skilled reader of the application. However, as noted above, there must not be any ambiguity for a correction to be allowable. Otherwise, the skilled person would be confronted by the correction with subject matter that is not originally disclosed.

(8) The Applicant thus failed to show that a skilled artisan would immediately recognize how to replace the incorrect sequences (i.e., what the specific sequence of the leader peptide would be), with certainty.

(9) The Applicant's representative further asserted that similar requests for corrections would have been accepted by the EPO, pointing to EP 1 558 648 and EP 2 581 113. Regarding EP 1 558 648, the relevant protein sequences as well as the CDR sequences were derived by standard sequencing procedures (Example 2, WO 2004/035607 A2, submitted herewith). The antibodies were then claimed by reference to V_L and V_H sequences or to CDR sequences which is also standard in the art. Moreover, the original application disclosed at p. 29, I. 35-36 that *"heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody"*.

(10) The Applicant of EP 2 581 113, had pointed to page 67, paragraphs [0222] and [0223] of the application as filed (submitted herewith), disclosing that the presence of a signal sequence in a VL or VH amino acid

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sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies.

(11) Both applications referred to by the Applicant therefore discuss the occurrence of leader sequences or a clear identification of the mature antibody by comparison to known peptides. As pointed out above, the current application as filed is completely silent about leader sequences and does not provide any guidance for the skilled person how to clearly identify the claimed antibody. Therefore, these cases cannot be compared to the present one.

(12) Notably, with the request for correction of an obvious error, the Applicant in the EP 1 558 648 case submitted an expert declaration of Tom Vink (attached hereto). In the declaration, the expert explains the following (section 10 of the Vink declaration):

> 10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

(13) According to the statement of the expert, V_L kappa signal peptides typically have a length of 19 to 23 AAs and end with C, A, G or E, while human V_L kappa chains begin with A, D, V, N or E. We note that the number of possible combinations appears to be even larger, when taking Kabat *et al.* into regard (see above). However, if this reasoning is applied to the sequences disclosed in EP 3 167 888 as filed originally (see below) at least one alternative cleavage site to the one suggested by Alexion would be apparent to the skilled person, namely between amino acids 19 and 20 (WLR**GA**RC). The resulting leader peptide would be 19 amino acids in length, end with a G residue and the resulting mature V_L chain would begin with an A residue. Thus, all requirements that the skilled person would allegedly be aware of (according to the Vink declaration) would be fulfilled.

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SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

(14) This is clear evidence that the skilled artisan would in the present case at the very least be confronted with two alternatives, when recognizing that SEQ IDs NO: 3 and 4 of the current application as filed erroneously comprise a leader peptide. Under such circumstances, the correction under Rule 139 EPC suggested by the Applicant would inevitably add information that is not originally disclosed.

(15) We lastly note that the *Kabat et al.* reference, as submitted by the Applicant, represents only a small (and potentially purposeful) selection from an amino acid sequence compendium that itself represents only a selection from the vast space of protein, or antibody sequences. It cannot provide evidence that nothing else than the sequences disclosed therein would have been thinkable sequences of signal peptides, or mature human kappa light chains for the skilled person.

(16) This is aggravated by the fact that the 5th edition of Kabat *et al.* was published in 1991, i.e., 15 years prior to the priority date of the application. In the quickly moving field of pharmacologically active antibodies, the space of known antibody sequences has significantly expanded within this period and thus, the sequence compendium referred to by the Applicant was long outdated at the priority date. The number of mature light chain sequences known in the art was certainly much larger in 2006, compared to 1991, accordingly, the number of possible options for the length and/or sequences of signal peptides was likely much larger in 2006, compared to 1991. Thus, the skilled person would have been confronted with even more uncertainty regarding the sequence and length of the signal peptide comprised in SEQ ID NOs: 3 and 4, even if recognizing that the sequences disclosed therein are immature.

(17) Thus, Kabat *et al.* is anyway not representative of the entire space of possible sequences the skilled person would have had to consider at the relevant date.

(18) In summary, the application provides no suggestion that specifically residues 1-22 of SEQ IDs NO: 3 and 4 are necessarily leader sequences, let alone that nothing else would have been intended than what the Applicant offered as correction. It can by no means be assumed that it would have been apparent to the skilled person that the sequence submitted by the Applicant is the only conceivable replacement for the sequences originally disclosed.

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(19) Therefore, the Applicant's request for correction of the description and the reference to residues 23 to 214 of SEQ ID NO:4, introduced into claim 1 of the MR contravene Articles 123(2) and 76(1) EPC.

II. The Auxiliary Requests filed on July 5, 2019

(20) With the submission dated July 5, 2019, the Applicant has submitted three Auxiliary Requests, AR1 to AR3. The Auxiliary Requests not only fail to overcome the objections raised by the Examining Division, they also introduce further violations of the EPC. This is explained in further detail below.

II.1 Auxiliary Request 1

(21) AR1 defines the claimed antibody by reference to SEQ ID NOs 2 and 4, however, the claim language has been amended such that claim 1 relates to an *"antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4"* instead of *"comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4"*.

(22) This alternative claim language is a clear violation of Articles 76(1) and 123(2) EPC. The application as filed refers to an antibody comprising a heavy chain and a light chain consisting of SEQ ID NOs: 2 and 4, respectively (cf. application as filed at p. 5, li. 30 to 33). In contrast, claim 1 of AR1 refers to an antibody which comprises the heavy and light chain sequences "shown in" SEQ ID NOs 2 and 4. The Applicant thus effectively replaced "consisting of" by "comprising", which in itself represents a violation of Articles 76(1) and 123(2) EPC, as the terms are not freely interchangeable (**T 759/10**).

(23) Thus, while the application as filed (and previous claim 1) defines the antibody in a closed manner (heavy and light chains consisting of SEQ ID NOs 2 and 4), claim 1 of AR1 encompasses antibodies that comprise the sequences "shown in" SEQ ID NOs 2 and 4, but may, due to the open claim language, also comprise further sequence elements. For example, an antibody comprising a heavy and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 elongated by a few further amino acids at the C-terminus would fall under claim 1 of AR1. The original disclosure does not teach such modified antibodies.

(24) The alleged basis provided by the Applicant in the letter dated July 5, 2019 (original application, p. 4, li. 32 and p. 44) fails to disclose the subject matter of claim 1 of AR1. The description text of the parent application as filed and of the divisional application as filed are identically worded. Page 4, line 32 of the original application teaches that *"[i]n certain embodiments, said antibody is eculizumab"* (emphasis added). This passage neither provides basis for the "comprising" language, nor for the vague "shown in" phrase of claim 1 of AR1. Page 44 of the application discloses the (partially incorrect) SEQ IDs. Taken together, these two passages indicate to the skilled person that the antibody disclosed in the application may be an antibody named "eculizumab", which consists of heavy chains and light chains, wherein the

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heavy and light chains consist of the sequences disclosed as SEQ ID NOs 2 and 4. A widening of this concept through the use of "comprising" language cannot be based on this disclosure.

(25) In addition, the introduction of the term "shown in" leads to a further violation of Articles 76(1) and 123(2) EPC. This is because different interpretations of this phrase are possible, at least some of which would introduce subject matter that lacks original disclosure. If *"the light chain amino acid sequence shown in SEQ ID NO: 4"* means "the light chain amino acid sequence of SEQ ID NO: 4", claim 1 of AR1 would refer to an antibody that is not eculizumab, and thus, an antibody for which protection is not actually sought, as explicitly stated by the Applicant (e.g., letter of July 5, 2019, p. 15, 1st full par.). The amendment would furthermore not solve the objections of the ED with regard to insufficiency of disclosure.

(26) Alternatively, the term "shown in" could be interpreted as meaning any light chain sequence(s) comprised in SEQ ID NO: 4. It may be assumed that this is the interpretation desired by the Applicant, because this would enable him to *inter alia* cover the (correct) light chain sequence of eculizumab via reference to the (incorrect) sequence disclosed in the application text (SEQ ID NO: 4). However, in this case, too, the amendment would not solve the objections of the ED with regard to insufficiency of disclosure, since a light chain amino acid sequence of SEQ ID NO: 4 would still be encompassed. Further, since SEQ ID NO: 4 encompasses at the very least two potential light chain sequences when interpreted in light of common general knowledge (cf. above), this claim interpretation would inevitably lead to the finding that claim 1 adds matter, since it now encompasses several (potential) light chain sequences, instead of one clearly defined light chain sequence.

(27) The introduction of the vague term "shown in" furthermore renders claim 1 wholly unclear. As explained above, it is not clear to the skilled person which exact sequence(s) are encompassed by claim 1 of AR1. The heavy and light chain sequences of SEQ ID NOs 2 and 4, or any heavy and light chain sequence(s) that are part of SEQ ID NOs 2 and 4, i.e., any sequence fragment that can be extracted from SEQ ID NOs 2 and 4 and that may serve as an antibody heavy, or light chain, respectively - together with further derivatives thereof comprising additional amino acids?

(28) In summary, AR1 violates at least Articles 84, 76(1) and 123(2) EPC.

II.2 Auxiliary Request 2

(29) Claim 1 of AR2 defines the claimed antibody by reference to *inter alia "residues 23 to 214 of SEQ ID NO: 4"*. As explained above, this reference to only part of SEQ ID NO: 4 is not derivable from the application as filed. Claim 1 of AR2 therefore suffers from the same deficiencies under Article 76(1) and 123(2) EPC as claim 1 of the Main Request.

II.3 Auxiliary Request 3

(30) AR3 is based on AR1, with claims 1 and 2 being reformulated to medical use claims by addition of the phrase *"for use in treating paroxysmal nocturnal hemoglobinuria"* to claims 1 and 2. Claim 2 is further

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amended by introduction of the definition of the antibody recited in claim 1. AR3 inherits the deficiencies under Article 76(1), 123(2) EPC and 84 EPC put forward above with regard to AR1.

Encl.
EP1558648_Request for correction
EP1558648_Original filing (WO 2004/035607 A2)
EP1558648_Vink declaration
EP2581113_Request for correction
EP2581113_Application as filed

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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	SK6930

	Description of document	Original file name	Assigned file name
1	Observations by third parties (Art. 115 EPC)	20190904-EP 3 167 888-Third Party observation.pdf	TIPA1-1.pdf
2	Observations by third parties (Art. 115 EPC)	TPOs EP 3 167 888.pdf	TIPA1-2.pdf
3	Citations filed by a third party	EP1558648_Original filing (WO2004035607 A2).pdf	TDOC-1.pdf
4	Citations filed by a third party	EP1558648_request for correction.pdf	TDOC-2.pdf
5	Citations filed by a third party	EP1558648_Vink Declaration.pdf	TDOC-3.pdf
6	Citations filed by a third party	EP2581113_Application as filed.pdf	TDOC-4.pdf
7	Citations filed by a third party	EP2581113_request for correction.pdf	TDOC-5.pdf

	Payment	
1	Method of payment	Not specified

Signatures

Place:

Date: **04 September 2019**

Signed by: **Rafal Witek 8336**

Representative name: **Rafał Witek**

Capacity: **(Representative)**

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European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	7825960
Application number	EP16195355.9
Date of receipt	04 September 2019
Receiving Office	European Patent Office, The Hague
Your reference	SK6930
Applicant	All applicants as on file

Documents submitted	
package-data.xml	ep-sfd-request.xml
epf1038.pdf (1 p.)	TIPA1-1.pdf\20190904-EP 3 167 888-Third Party observation.pdf (1 p.)
TIPA1-2.pdf\TPOs EP 3 167 888.pdf (7 p.)	TDOC-1.pdf\EP1558648_Original filing (WO2004035607 A2).pdf (201 p.)
TDOC-2.pdf\EP1558648_request for correction.pdf (3 p.)	TDOC-3.pdf\EP1558648_Vink Declaration.pdf (17 p.)
TDOC-4.pdf\EP2581113_Application as filed.pdf (129 p.)	TDOC-5.pdf\EP2581113_request for correction.pdf (5 p.)

Submitted by	CN=Rafal Witek 8336
Method of submission	Online
Date and time receipt generated	04 September 2019, 13:48 (CEST)
Message Digest	B7:3C:D9:F2:08:DC:77:B3:13:54:36:4C:71:5A:F5:66:DD:8F:F2:11

Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Date
06-09-2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication pursuant to Rule 114(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115 EPC).

Under Rule 114(2) EPC you may comment on the observations.

For the Examining Division

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URGENT! PLEASE FORWARD IMMEDIATELY TO THE EXAMINING DIVISION!
ORAL PROCEEDINGS SCHEDULED FOR SEPTEMBER 5, 2019

September 2, 2019

Third Party Observations

Application Number	16 195 355.9 - 1466
Publication Number	EP 3 167 888
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Applicant	Alexion Pharmaceuticals, Inc.
Our file reference	AMG17203ADV-EP888

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

The Applicant acted against the well-established principle of *nemo potest venire contra factum proprium*:

(1) In their submission dated August 15, 2018, Alexion attempt to defend their contradictive behavior in the proceedings regarding EP 1 720 571 and the current application. The Applicant's representative asserts that the principle of *nemo potest venire contra factum proprium* would not be applicable in this case (cf. p. 6, ultimate par. of submission dated August 15, 2018). This is incorrect.

(2) The principle of the protection of legitimate expectations is a general principle well established in European Community law and generally recognized in the EPC contracting states and Board of Appeal case law (**G 2/97**, OJ 1999, 123; see also **R 4/09**). The term "good faith" is also used to describe this concept (**J 10/84**, OJ 1985, 71; **J 38/97**). Hence, it is established case law that users of the European patent system, who are

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H. Keith Withers [1] Ph.D., M.Sc. Biochem., LL.B.
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Partnerschaftsregister des AG München
PR-Nr. 1278

parties in proceedings before the EPO, must act in good faith (**G 2/97**, **R 4/09**).

(3) According to the "Saxe Declaration" submitted by the Applicant on January 16, 2018, Stephen Saxe submitted a letter to CAS dated April 28, 2009 explaining:

> *"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."*

(4) Thus, Alexion were aware that they provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Contrary to the Applicant's assertion, when filing the response of December 30, 2013 in the EP'571 case, Alexion thus did not make a *"mere assertion that turned out to be erroneous"* (cf. p. 6, 4th par. of submission dated August 15, 2018). Rather, they made an assertion and at the same time apparently assumed that the contrary was the case.

(5) In their submission dated July 5, 2019, Alexion argue that the copy of the CAS entry, provided with their letter in the opposition proceedings regarding EP 1 720 571 did not contain any sequences, that the identity of the sequences was not investigated, and that the argument was not challenged at that time (cf. p. 6, 6th to 9th par. of letter dated July 5, 2019). We note that none of these allegations take away from the fact that Alexion have asserted before the OD in the EP'571 case that the sequence of eculizumab had been submitted to CAS knowing that this was incorrect.

(6) Alexion further try to defend themselves by arguing that they did not gain any advantage from their knowingly false statement (cf. p. 7, 1st par. of letter dated July 5, 2019). Of course, the obligation to act in good faith does not depend on the question whether a party gains an advantage or not.

(7) On the other hand, Alexion did not provide any evidence that they indeed filed the wrong structural information to CAS, since the Saxe Declaration - contrary to the assertion of Alexion's representative - does not include said "wrong" structure or any excerpt of the CAS registry showing that a "wrong" structure was submitted there. With the letter dated July 5, 2019, Alexion filed an excerpt from a CAS online resource allegedly showing the sequences originally submitted to CAS (**D28**). We note that the document provided by the Applicant does not include any information about the date on which the sequence shown therein has been submitted to CAS and/or been amended. This document, apparently retrieved on June 11, 2019, is therefore

unable to prove that the incorrect sequence has been submitted to CAS in 1999, i.e., twenty years ago.

(8) In this regard we would like to point out that the Applicant's allegation – that the patents and applications cited in **D8** would refer to anti-C5 antibodies more generally and that it would, therefore, be of no importance whether or not the correct sequence of eculizumab had been disclosed (p. 8, 1st to 3rd full paragraph of Applicants submission of August 15, 2018) - is simply not tenable. Rather, the question whether or not the correct sequence of the antibody designated via the INN eculizumab was available at CAS – published and provided with a CAS registry number - was of utmost importance.

(9) An INN is an **official** generic and non-proprietary **name** given to an active ingredient. The INN "eculizumab" is the **official** generic **name** given to the substance having the CAS-Number 219685-50-4. In conclusion, the above presented facts inevitably imply that the public – in particular authors of scientific literature but also the EPO - could rely upon the fact that a correct description of eculizumab was publicly available at the priority date.

(10) As will be explained below, during prosecution of EP 1 720 571 B1 and EP 2 359 834 B1, the Examining Division considered the term "eculizumab" at all times to disclose the specific antibody related to said INN. Alexion's representatives took the same position, even though Alexion apparently assumed at least as of April 2009 that the INN eculizumab related to a false structure (as they allege now).

(11) Claim 1 of EP 1 720 571 B1 as granted directly relates to eculizumab. It is readily derivable from the prosecution history (see first office action dated September 2, 2009 (enclosed as **Annex I**), Items 4.1 to 4.3 of the Communication; Summons to Oral Proceedings dated March 4, 2011 (enclosed as **Annex II**), Items 2.1, 5.1, 6.1) that the Examining Division heavily relied upon the fact that eculizumab was known on the priority date. In the Summons to Oral Proceedings the Examining Division further stated under Item 3.2 that:

> *"The application provides experimental details of dosage optimization of an anti-C5 antibody, 5G1.1* ***eculizumab****, for the treatment of PNH in a group of 11 patients.* ***Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used****. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention."*

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(12) Although Alexion assumed that eculizumab was not disclosed at the priority date, as they now allege, the disclosure of eculizumab was confirmed by them vis-à-vis the Examining Division (see reply to Summons to Oral Proceedings dated September 23, 2011 (enclosed as **Annex III**), page 9, second paragraph). Because the application as filed did not disclose the actual structure of eculizumab this was the only way to overcome the cited objection.

(13) The sole claim of EP 2 359 834 B1 as granted directly relates to eculizumab. Again, the Examining Division heavily relied upon the notion that eculizumab had been disclosed before the priority date (see e.g. European Search Opinion dated July 21, 2011 (enclosed as **Annex IV**), objection under Item 2, upheld by the Examining Division in further prosecution). The prior disclosure of eculizumab was also confirmed by Alexion *vis-à-vis* the Examining Division (see reply to Office Action dated November 15, 2012 (enclosed as **Annex V**), Item 2.2.2):

> *"This understanding is further underlined by the general knowledge in the art of antibody engineering and the skilled person's understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18; see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as Exhibit A)). Applicant also provides Mueller et al. (1997) Mol Immunol 34(6):441-452 as Exhibit B, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9)."* (Emphasis added.)

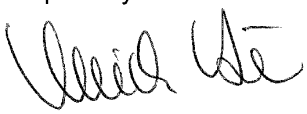
(14) Again, Alexion relied upon the public's knowledge of the structure of eculizumab before the Examining Division to support the patentability of claims to "eculizumab" but now assert that one of skill in the art lacked such knowledge.

(15) In summary, the WHO named eculizumab to be the official generic name given to the substance having the CAS-Number 219685-50-4. According to their own statements, Alexion were aware of having provided incorrect information regarding eculizumab to CAS at least as of April 28, 2009. Therefore, the concealment of this information cannot be considered to merely have been erroneous. By concealing this information Alexion cannot be said to have acted in good faith when attempting to overcome the various objections from the Examining Division, which objections could not have been overcome had the EPO been informed that the INN eculizumab was in fact assigned to a false structure. On the other hand, there is no evidence provided that Alexion have indeed filed "wrong" structural information to CAS. At the moment, this appears to be a mere assertion.

(16) In conclusion, Alexion's conduct vis-à-vis the EPO is contrary to the principle of *nemo potest venire contra factum proprium*, and their attempt to justify their concealment of alleged facts, which, if true would have been critical for validity in a number of proceedings, and which are

nonetheless now supposed to serve as basis to restore novelty over prior art documents, must be refused.

Respectfully



Dr. H. Ulrich Dörries

Enclosures
Annexes I-V

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The examination is being carried out on the **following application documents**:

Description, Pages

1-39 as published

Claims, Numbers

1-27 filed with entry into the regional phase before the EPO

Drawings, Sheets

1/11-11/11 as published

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure:

D1: ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia." HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825 ISSN: 1520-4391

D2: JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough" 19000101, [Online] 13 December 2002 (2002-12-13), pages 1-2, XP009106338 Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle /446336> [retrieved on 2008-09-24]

D3: "Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria" TRANSPLANT NEWS, [Online] 15 January 2004 (2004-01-15), XP002497241 Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_ m0YUG/is_1_14/ai_n17208612> [retrieved on 2008-09-25]

D4: HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)" BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083 ISSN: 0006-4971

D5: THOMAS T C ET AL: "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv" MOLECULAR IMMUNOLOGY, ELMSFORD, NY, US, vol. 33, no. 17-18, 1 January 1996 (1996-01-01), pages 1389-1401, XP002262113 ISSN: 0161-5890

D6: HILLMEN PETER ET AL: "Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria." THE NEW ENGLAND JOURNAL OF MEDICINE 5 FEB 2004, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948 ISSN: 1533-4406

D7: FITCH JC ET AL: 'Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass' CIRCULATION vol. 100, no. 25, December 1999, pages 2499 - 2506, XP002209328

D8: DATABASE PUBMED 16464755 HILL A ET AL: 'Improvemnet in the symptons of smooth muscle dystonia during eculi therapy in paroxysmal nocturnal hemoglobinuria', XP003000273

1.0 Introduction

The present application relates to treatments for the effects of hemolytic disease using an anti-C5 (complement component C5) antibody (h5G1.1; Eculizumab). A small study indicates also that Eculizumab treatment in combination with administration of erythropoietin can reduce transfusion requirements of thrombocytopenic patients, albeit only for those with a good marrow reserve.

2.0 Article 123(2) EPC

The amended claims 1-27 are considered allowable under Article 123(2) EPC.

3.0 Article 84 EPC

3.1 Claims 1-5,8-10,12-14,16 and 17 do not meet the requirements of Article 84 EPC in that the matter for which protection is sought is not clearly defined. The following functional statements do not enable the skilled person to determine which technical features are necessary to perform the stated functions:

"Use of a molecule selected from the group consisting of (a) molecules which bind to one or more complement components, (b) molecules which block the generation of one or more complement components, and (c) molecules which block the activity of one or more complement components for ... "

The suggested grouping is so broad as to include a variety of molecules, of which it is not clear whether all would indeed be suitable for the desired use. For example, there are several known antibodies that can bind complement, but which do not modulate its function. Furthermore, molecules that interfere with members of the complement cascade prior to C5 may create a vulnerability to both pyogenic infections and autoimmune disease and thus would not be suitable (See document D1, Fig 4).

3.2 Paroxysmal nocturnal hemoglobinuria (PNH) is characterised by hemolysis, hemoglobinuria, thrombosis, anemia, erectile dysfunction, esophageal spasm (dysphagia), and abdominal pain (see p2, para.2 of the description). It could therefore be expected that any treatment for PNH should relieve some of these symptoms. However, the formulation of present claims 2-5,8 and 16 implies that the invention is applicable for the treatment of these symptoms in all patients. Present claims 8 and 9, for example, refer to a composition for increasing the proportion of type III red blood cells of a subject`s total red blood cell count, however, it is not clear from the application whether the composition claimed would be effective in all humans or just those with hemolytic disease.

3.3 The definition of the term "hematopoiesis" defined in the description (p7, li. 22-25) and referred to in claims 13,14,26 and 27 shall be taken to be that commonly accepted in the field of hematology i.e. the formation of blood cells in the body. Thus, substances which simply prolong the life of blood cells will not be considered to be included under this term.

3.4 Further medical use claims 1,8,12 and 17 are not acceptable under Article 84 EPC. The therapeutic application is functionally defined by a mechanism of action which does not allow any practical application in the form of a defined, real treatment of a pathological condition (disease) (see Guidelines C-IV, 4.8).

The objection could be overcome by either introducing into the claims a list of

pathological conditions (diseases) cited in the application, or by showing that means are available which would allow the skilled person to recognise which additional condition/s would fall within the functional definition (see Guidelines C-III, 6.5).

3.5 Claims 1-5,8,10,12,13,16 and 17 have been drafted as separate independent claims.

Under Article 84 in combination with Rule 43(2) EPC, an application may contain more than one independent claim in a particular category only if the subject-matter claimed falls within one or more of the exceptional situations set out in paragraph (a), (b) or (c) of Rule 43(2) EPC. This is not the case in the present application, however, for the following reasons: the inventions as claimed are not obviously related.

In the further prosecution of the application, failure to file an amended set of claims which complies with Rule 43(2) EPC, or to submit convincing arguments as to why the current set of claims does in fact comply with these provisions, may lead to refusal of the application under Article 97(2) EPC.

4.0 Article 54 EPC

Furthermore, notwithstanding the above-mentioned lack of clarity, the subject-matter of claims 1-12 and 16-24 is not new within the meaning of Article 54(1) and (2) EPC, and therefore the requirements of Article 52(1) EPC are not met.

4.1 Document D1 discloses that humanised monoclonal antibody against complement C5 has "a remarkable effect in controlling the manifestations of [hemolytic] disease". For example, paroxysmal nocturnal hemoglobinuria (PNH) is characterised by hemolysis, hemoglobinuria, thrombosis, anemia (p48, col.1, last para.), erectile dysfunction, esophageal spasm (dysphagia), and abdominal pain (p49, col.1 lines 31-33). The latter two resulting from reduced NO, since in PNH, released hemoglobin scavenges NO thereby reducing free NO in the blood (p49, col.2, last para). The document describes how the anti-complement C5 mAb,

Eculizumab, blocks the creation of the membrane attack complex, preventing the release of the potent anaphylatoxin C5a and hemolysis (and thus hemoglobin release). Since Eculizumab treatment leads to an increase in the proportion of PNH red cells of 36.7-59.2% (p53, col.1, lines 40-46) patients with hemolytic disease may therefore cease or require less frequent transfusions. This document also mentions the use of soluble, membrane-anchorable CD59 to prevent hemolysis (p53, second para.).

4.2 Document D2 is a report from 13.12.2002 based on Abstract 154 presented on 9.12.2002 at the 44th Annual Meeting of the American Society of Hematology (ASH) and refers to the use of Eculizumab for the treatment of the symptoms of PNH, in particular hemolysis. The therapy reduced the need for blood transfusions by 68%.

4.3 Document D3 describes the use of anti-complement C5 mAb (Eculizumab), corticosteroids, immunosuppressive drugs, and blood transfusions as treatments for the symptoms of PNH.

Documents D1-D3 therefore all anticipate the novelty of the present claims 1-12 and 16-24

4.4 Document D7 discloses the use of anti-human complement C5 (h5G1.1-scFv; Pexelizumab) for the treatment of hemolytic disease (p2504, col.1, li.20-28) and by implication its associated symptoms.

Document D7 thus anticipates the novelty of claims 1,8,12 and 17-24

5.0 Article 56 and 83 EPC

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 6,13-15,18,21 and 25-27 is not sufficiently disclosed according to Article 83 EPC and/or does not involve an inventive step within the meaning of Article 56 EPC.

5.1 Although the description provides data for the use of the anti-C5 antibody

Eculizumab and EPO for treating the effects of hemolytic disease, the use of other molecules grouped in claims 6,14,18,21 and 27 is not disclosed (Article 83 EPC). Given that the other molecules listed lack any similarity to anti-C5 mAb and EPO it cannot be accepted that they also provide solutions for the treatment of the disclosed symptoms. Claims 6,14,18,21 and 27 therefore lack an inventive step (Article 52(1) and 56 EPC).

5.2 The subject matter of present claims 13-15,26 and 27 relates to routine variations that cannot be seen as involving an inventive step according to Article 56 EPC.

5.3 The timing of transfusion independence (present claim 25) cannot be considered to be inventive under Article 56 EPC since there is no evidence in the present application suggesting that the claimed technical effect can be achieved by any other substance to that known in the prior art i.e. anti-C5 mAb.

6.0 Further remarks

The Applicant is informed that a non-unity objection could have been raised in view of the claims as presently formulated since the unifying features: a) linking the different diseases to be treated is considered to be based in that they are all somehow linked to PNH; and b) linking the different alternative molecules which bind/block complement is considered to be based on that they are suitable for treating PNH, are both known in the art (see p2, para.2 of the description, Documents D1-D3).

However, due to the insufficiencies as indicated above under items 3-5 which demand suitable amendments, no objection was held at the search stage, but depending on the amendments made, might become necessary at a later stage.

The examination is being carried out on the **following application documents**

Description, Pages

1-39	as published

Claims, Numbers

1-14	received on	31-01-2011	with letter of	28-01-2011

Drawings, Sheets

1/11-11/11	as published

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia.", HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825, ISSN: 1520-4391

D2 JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough", 19000101, [Online] 13 December 2002 (2002-12-13), pages 1-2, XP009106338, Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle/446336[retrieved on 2008-09-24]

D3 "Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria", TRANSPLANT NEWS, [Online] 15 January 2004 (2004-01-15), XP002497241, Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_m0YUG/is_1_14/ai_n17208612 [retrieved on 2008-09-25]

D4 HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083, ISSN: 0006-4971

D5 THOMAS T C ET AL: "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv", MOLECULAR IMMUNOLOGY, ELMSFORD, NY, US, vol. 33, no. 17-18, 1 January 1996 (1996-01-01), pages 1389-1401, XP002262113, ISSN: 0161-5890

D6 HILLMEN PETER ET AL: "Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria.", THE NEW ENGLAND JOURNAL OF MEDICINE 5 FEB 2004, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406

The following documents are cited by the Examiner. A copy of the documents is annexed to the communication and the numbering will be adhered to in the rest of the procedure.

D9 HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH).", BRITISH JOURNAL OF HAEMATOLOGY, vol. 121, no. Supplement 1, May 2003 (2003-05), page 87, XP009144758, & 43RD ANNUAL SCIENTIFIC MEETING OF THE BRITISH SOCIETY FOR HAEMATOLOGY; GLASGOW, UK; APRIL 07-09, 2003 ISSN: 0007-1048

D10 KAPLAN M: "ECULIZUMAB", CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 1017-1023, XP009045356, ISSN: 1472-4472

1 Introduction

1.1 The Applicant has elected the further examination of invention 4, as set out in our previous communication following an objection under Article 82 EPC.

1.2 The Examining Division considers oral proceedings (pursuant to the request in the Applicants' letter of 28.01.2011) as expedient because the Applicants' arguments are not deemed persuasive.

Oral proceedings are hereby appointed.

Matters to be discussed at oral proceedings

2 Article 123 EPC

The amended claims 1-3,5-10 and 11-14 are not considered allowable under Article 123(2) EPC.

2.1 The only basis provided for the specific dose regimes of claims 1-14 relates to antibody 5G1.1, i.e. eculizumab, and not anti-C5 antibodies in general.

2.2 Claims 1-3, and 8-10 further refer to 'serum complement activity' however, p. 38. li.13 describes only 'cell lysis' being reduced to below 20% of normal serum complement activity.

2.3 Claims 2 and 9 relate to a specific dosage regime, however, no basis can be found that **this specific dosage regime** resulted in the 'cell lysis' being reduced to below 20% of normal serum complement activity. As far as can be ascertained from p.35, li.5-22, only two patients received this dosage schedule. Only in patients receiving 900mg of eculizumab '**every other week**' (p.36, li.18) was 'cell lysis' seen to be reduced to below 20% of normal serum complement activity.

2.4 There is no basis for the general use of an increased dose or increased frequency of dose as stated in claim 3. A basis can only be seen to exist for administering 900mg every 12 days. Furthermore, there is no basis for these features in combination with antibody serum combination and serum complement activity.

2.5 Claim 10 referring to an amount effective to maintain a serum concentration of antibody around 35 µg/ml has no basis in the description. Basis only exists for the administration of **900mg eculizumab every 12 days** to maintain levels **above** 35µg/ml.

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3 Articles 83 and 84 EPC

The application does not meet the requirements of Articles 83 and/or 84 EPC, because claims 1-3 and 8-10 are not clear, and/or lack support and disclosure.

3.1 Rule 43(2) EPC states that a European patent application may only contain more than one independent claim in the same category if the subject matter of the application relates to either: a plurality of interrelated products; different uses of a product; or alternative solutions to a particular problem, where its is inappropriate to cover these alternatives by a single claim. In the present case, however, claims 1-3 and 8-10 all embody related to solutions to the same problem, i.e. the provision of a dosage schedule for PNH.

3.2 The application provides experimental details of dosage optimisation of an anti-C5 antibody, 5G1.1 eculizumab, for the treatment of PNH in a group of 11 patients. Given the different affinities, functions and pharmacokinetics of individual, specific antibodies, the establishment of an optimal dosage schedule will be dependent on the antibody used. Since independent claims 1-3 and 8-10 do not contain this feature it does not meet the requirement following from Article 84 EPC, taken in combination with Rule 43(1) and (3) EPC, that any independent claim must contain all the technical features essential to the definition of the invention. A lack of support and disclosure therefore occurs for present claims 1-3 and 8-10 over their broad scope.

3.3 Claims 3 and 10 do not meet the requirements of Article 84 EPC in that the matter for which protection is sought is not defined. The claim attempts to define the subject-matter in terms of the result to be achieved. Such a definition is only allowable under the conditions elaborated in the Guidelines C-III, 4.10. In this instance, however, such a formulation is not allowable because it appears possible to define the subject-matter in more concrete terms, viz. in terms of how the effect is to be achieved, i.e. the administration schedule required.

3.4 The relative term 'about' used in claim 10 has no well-recognized meaning and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 84 EPC).

4 Priority

The priority document does not appear to disclose the subject matter of the present claims 3 and 10, relating to the dosing schedules for the maintenance of a serum concentration of antibody above 35 µg/ml. The P document D6 is therefore citable as prior art.

5 Article 54 EPC

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 3 and 10 is not new within the meaning of Article 54(1) and (2) EPC.

5.1 D6 discloses the use of a 900mg dose of eculizumab every 12 days for the maintenanceof a serum concentration of 35 µg/ml (p.555, col.1, §4 - p.556, col.1, §1).

D6 thus anticipates the subject-matter of present claims 3 and 10.

6 Article 56 EPC

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1-14 does not involve an inventive step within the meaning of Article 56 EPC.

6.1 Document D2 is considered to be the closest prior art to the subject-matter of claim 1 and discloses a trail for the treatment of PNH, comprising administering 600mg eculizumab/patient/week for four weeks, followed by 900mg/patient every two weeks.

The subject-matter of claim 1 therefore differs from this known D2 in that the same dose is administered every 12 days.

The technical effect resulting from this difference would appear to be the prevention of relapse in 10% of the patients where hemolytic activity returned.

The problem to be solved by the present invention may therefore be regarded as the optimisation of a known dosing schedule for individual patients.

The solution proposed in claim 1 of the present application cannot be considered to involve an inventive step (Articles 52(1) and 56 EPC), for the following reasons:

The use of eculizumab for the treatment of PNH is well known in the prior art (see D1-D4 and D9) as well as useful dosage regimes (D2 and D9). It would therefore be obvious to the skilled person with a knowledge of present dosage regimens for PNH described in D2 and D9, and presented with a patient

displaying relapse, to adjust the dosage regimen through increased dose or frequency. In particular, it was already known from D10 that a single dose of eculizumab could block complement activity for **up to** two weeks (p.1019, col. 2, §2), therefore 14 days would be considered an extended interval that the skilled person would reduce upon observing relapse.

The dose regimen claimed is therefore considered to constitute a routine improvement with no new surprising effect.

6.2 For the same reasons as stated above (see, 6.1), claims 2,3 and 8-10 can also not be considered inventive.

6.3 Dependent claims 4-7 and 11-14 do not appear to contain any additional features which, in combination with the features of any claim to which it refers, meet the requirements of the EPC with respect to novelty and/or inventive step.

7 Concluding Remarks

7.1 The Applicant may file new claims which take in to account the above comments, however, on the basis of the present application documents, refusal of the application under Article 97(2) is to be expected. The Applicants' attention is therefore drawn to the possibility of requesting an appealable decision according to the state of the file (Guidelines E-X, 4.4).

7.2 In order to facilitate the examination of the conformity of any amendments to the application with the requirements of Article 123(2) EPC, the Applicant is requested to clearly identify such amendments, irrespective of whether they concern amendments by addition, replacement, or deletion, and to indicate the passages of the application as filed on which these amendments are based.

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a **printed** copy of the relevant parts of the application as filed.

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23. Sep. 2011

EP 05 71 2607.0 - 2406
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

München, September 23, 2011
WA/ARO/FLG

This is in reply to the Summons to attend oral proceeding issued in the above-mentioned case. The indication of the chairman to accept written submission until September 23 2011 is greatly appreciated.

Enclosed is a main request, a new first auxiliary request, a new second auxiliary request, a new third auxiliary request and a new fourth auxiliary request. These requests replace the previously filed claim set.

1. AMENDMENTS TO THE CLAIMS (Rule 137(4) EPC)

1.1. Main Request and Auxiliary Request I to III

Support for the amendments to the claims constituting the first auxiliary request, the second auxiliary request, and third auxiliary request is indicated in the following Table.

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New Claim #	Previous claims and passages in the description supporting the Main Request	Previous claims and passages in the description supporting the Auxiliary Request 1	Previous claims and passages in the description supporting the Auxiliary Request 2	Previous claims and passages in the description supporting the Auxiliary Request 3
1	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1 and page 38, lines 11-17	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and page 37, line 10 to page 38, line 16	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claim 4	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claims 1 and 4
2	1	1	1	2
3	2	2	2	3
4	3	3	3	5
5	page 37, line 10 to page 38, line 16	5	page 37, line 10 to page 38, line 16	6
6	5	6	5	7
7	6	7	6	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claims 8 and 11
8	7	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and page 37, line 10 to page 38, line 16	7	9
New Claim #	Corresponding previous claims/ support for Main	Corresponding previous claims/ support for Auxiliary 1	Corresponding previous claims/ support for Auxiliary 2	Corresponding previous claims/ support for Auxiliary 3

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9	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1 and page 38, lines 11-17	8	page 5, lines 6 to 10; page 4, line 20 to page 5, line 1; page 38, lines 11-17 and claim 11	3
10	8	9	8	12
11	9	3	9	13
12	3	12	3	14
13	page 37, line 10 to page 38, line 16	13	page 37, line 10 to page 38, line 16	
14	12	14	12	
15	13		13	
16	14		14	

1.2. Auxiliary Request IV

1.2.1. New claim 1 is based on claim 1 as previously filed. The term " to thereby maintain in the patient a serum complement activity at a level below 20% of the normal serum complement activity" has been deleted. The anti-C5 antibody has been further defined as being eculizumab (i.e. also designated as antibody h5G1.1 in the application). The deletion of said term and the use of eculizumab are supported at page 4, line 17 to page 5, line 5 of the application as filed.

The term "at least" has been deleted and the administration has been further defined as intravenous administration. Support for the dosage of eculizumab recited in new claim 1 has been indicated in section 1.2. of our submission of January 28, 2011. Further support can be found at page 35, lines 5-22 of the application as filed.

1.2.2. New claims 2 to 4 correspond to claims 5 to 7 as previously filed.

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1.2.3. New claims 5 to 8 are based on new claims 1 to 4 and have been rephrased in the "Swiss-type" claim format. In accordance with decision G 1/09 the applicant is allowed to also use the "Swiss-type" claim format in the present application.

2. INADMISSIBLE BROADENING (Article 123(2) EPC)

2.1. Antibody 5G1.1

2.1.1. *Main Request*

At page 3 of the Office Action, claims 1-3 and 5-14 were objected to as allegedly lacking written description. According to the Examining Division, "the only basis provided for the specific dose regimes of claims 1-14 relates to antibody 5G1.1, i.e. eculizumab, and not anti-C5 antibodies in general." Applicant disagrees.

The instant claims, as amended, are drawn to novel, inventive, and fully-supported uses of an anti-C5 antibody to prevent serum complement activity breakthrough in a patient afflicted with PNH. *See* specification at, e.g., page 5, lines 6 to 10. *See also* specification at page 4, line 20 to page 5, line 1 (describing use of anti-C5 antibodies such as eculizumab and pexelizumab). For example, claim 1 of the Main Request is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity.

As acknowledged by the Examining Division, the specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal. These particular approaches are embraced by claims 2, 3, 10, and 11, as well as the claims dependent therefrom; accordingly, these claims are believed in view of the remarks of the Examining Division to *per se* satisfy the requirements of Article 123 EPC.

Yet, aided by the express teachings of the specification, a skilled artisan at the effective filing date of the application would have appreciated that there are a number of therapeutic anti-C5 antibodies and associated therapeutic approaches

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that could be used to prevent serum complement activity breakthrough in PNH patients as claimed. In fact the specification describes pexelizumab in addition to eculizumab. With the benefit of the teachings of the specification, the skilled artisan would have understood that other therapeutic regimens (dosing amounts and intervals) were available for the claimed use with pexelizumab – as well as other anti-C5 antibodies – to maintain serum complement activity of PNH patients at a level below 20% of normal complement activity.

In view of the foregoing, it is clear that the skilled artisan would have at once unambiguously appreciated that Applicant provided in the as-filed specification a clear basis for the entire scope of the subject matter claimed. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

2.1.2. *Auxiliary Requests II to IV*

Auxiliary Requests II to IV recite "eculizumab" in all of the claims, thus, rendering the rejection moot.

2.2. <u>The term "a serum complement activity level below 20% of normal serum complement activity"</u>

2.2.1. Claims 1-3 and 8-10 were also objected to as allegedly being drawn to new matter. Specifically, the Examining Division urges that the specification does not provide descriptive support for "serum complement activity...being reduced to below 20% of normal serum complement activity." Applicant disagrees.

2.2.2. The instant claims involve the use of an anti-C5 antibody to maintain in PNH patient serum a complement activity at a level below 20% of the normal serum complement activity. Applicant respectfully points out the express support for this claim language at, e.g., page 38, lines 11-17 and Fig. 8 of the specification, in which the inventors describe the physiological effects of eculizumab treatment of PNH over time. Specifically, the specification states that "serum hemolytic activity (i.e. ...cell lysis)...was dramatically reduced in the majority of patients to below 20% of normal serum complement activity under eculizumab treatment." *Id.* Thus, as an initial matter, this passage makes clear that the inventors use the terms "serum complement activity", "cell lysis", and "serum hemolytic activity"

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interchangeably. The interchangeability of these terms becomes even more clear when viewed in the context of the experiments set forth in the application.

For example, at pages 37 and 38 of the specification, the inventors describe the results of experiments in which patient serum was tested for its complement activity as a function of its ability to lyse red blood cells (hemolysis) *in vitro*. This test, known as a hemolysis assay, involves incubating chicken erythrocytes with serum from healthy individuals (normal serum) or serum from eculizumab-treated PNH patients (patient serum). In the presence of normal serum that contains a full battery of complement component proteins, the chicken erythrocytes are lysed thereby releasing hemoglobin from the cells (as determined by optical density). By contrast, patient serum, in which eculizumab has, in effect, inhibited the activity of complement component C5, hemolysis is reduced and thus less hemoglobin is released. Following this assay, the inventors determined the percent hemolysis in patient serum as compared to normal serum essentially by dividing the amount of hemoglobin released from erythrocytes in patient serum (patient serum complement activity) by the amount of hemoglobin released from erythrocytes in normal serum (normal serum complement activity), multiplied by 100. The skilled artisan aware of hemolysis assays would have readily understood that "percent hemolysis" is just another way of expressing "percent cell lysis" or "percent serum complement activity" in patients as compared to corresponding normal human serum, the latter of which being recited in the claims of the Main Request.

2.2.3. In T 0878/09, the Board recently made clear that the Examining Division's

> "determination of whether or not the provisions of Article 123(2) EPC are fulfilled requires the determination of 'subject matter'." The Board further cautioned that "[s]ince the same subject-matter may be described by different words – for example the same glass may be described as either 'half full' or 'half empty' – a difference in wording does not per se necessarily establish that the subject-matter defined by the words is different."

In this case, the Examining Division objects to a linguistic distinction without a difference in meaning. The skilled artisan reading the instant application would have at once clearly and unambiguously appreciated that serum "cell lysis" and "serum complement activity", as used by the inventors, are equivalent terms. Thus, Applicant submits that the objected to claim language was fully defined

and described in the specification as filed such that a skilled artisan would have readily understood the metes and bounds of the claims. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

2.3. Dose as stated in claim 3

2.3.1. The Examining Division alleges that" [t]here is no basis for the general use of an increased dose or an increased frequency of dose as stated in claim 3."

2.3.2. Applicant respectfully disagrees. However solely in the interest of expediting prosecution of this application, the amendments to claim 3 (now claim 4) are believed to render the objection moot.

2.4. A serum concentration of antibody around 35 µg/ml

2.4.1. The Examining Division alleges that "[c]laim 10 referring to an amount effective to maintain a serum concentration of antibody around 35 µg/ml has no basis in the description."

2.4.2. Applicant respectfully disagrees. However solely in the interest of expediting prosecution of this application, the amendments to claim 10 (now claim 12) are believed to render the objection to claim 10 moot.

2.5. Auxiliary Request IV

The subject-matter of the claims of the fourth auxiliary request closely adheres to and reflects the results of the application as filed, in particular at page 32, lines 12-16 and page 35, lines 5-22 of the application as filed. Moreover, this request is confined to the dosage considered supported by the Examining Division.

3. SUFFICIENCY OF DISCLOSURE, CLARITY, CONCISENESS AND SUPPORT (Articles 83 and 84 EPC)

3.1. Independent claims of the same claim category (Rule 43(2) EPC)

3.1.1. At page 4 of the Office Action, the Examiner invoked Rule 43(2) EPC to object to the claims as having more than one independent claim in the same category.

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3.1.2. While Applicant does not concede to any aspect of the stated reasons for objection, the instant claims contain only two independent claims (claims 1 and 9), each of which is drawn to a different category, i.e., claim 1 (composition-for-use) and claim 9 (Swiss-type use). In accordance with decision G1/09 the applicant is allowed to use the format of a second medical use claim under the EPC 2000 and the "Swiss-type" claim format in the present application. The corresponding new claims 1 and 9 are the only independent claims.

In view of the instant amendments, the objection to the claims is rendered moot.

3.2. The antibody h5G1.1

3.2.1. *Main Request*

In this aspect of the Office Action, the Examiner again objects to the independent claims because they are not limited to eculizumab. According to the Examining Division, "[g]iven the different affinities, functions and pharmacokinetics of individual specific antibodies, the establishment of an optimal dosing schedule will be dependent on the antibody used." At bottom, the Examiner contends that, in contravention of Rules 43(1) and 43(3) EPC, the independent claims allegedly do not "contain all the technical features essential to the definition of the invention." Office Action at page 4. Applicant respectfully disagrees.

Rule 43(1) requires that the claims define the subject matter claimed in terms of "technical features which are necessary for the definition". Rule 43(3) further specifies that a broad claim setting forth the necessary technical features may be followed by additional claims drawn to particular embodiments of the broader claim. The instant claims amply satisfy these requirements.

As noted above, claim 1 is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity. This independent claim, as with its sister claim 8 in the Swiss-type use format, is defined by, among other things, structure, e.g., an anti-C5 antibody, and affirmative step – that the antibody is to be administered to a patient to maintain a

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serum complement activity level below 20% of the normal serum complement activity. The claim further defines the particular patient population – PNH patients – to which the uses are directed and particular pathological state – serum complement activity breakthrough – sought to be averted.

Under the guidance of these numerous essential features, a skilled artisan at the effective filing date of the application would have recognized, as did the inventors, that there are a number of therapeutic anti-C5 antibodies (e.g., eculizumab and pexelizumab) and associated therapeutic approaches that could be used to prevent serum complement activity breakthrough in PNH patients as claimed. The specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal.

In accordance with Rule 43(3), claims 2, 3, 10, and 11 of the Main Request further define particular embodiments of the broader claim, particularly the use of eculizumab under particular dosing regimens. The Examiner has provided no reason as to why a skilled artisan, in view of the essential technical features set forth in the claims, would not have readily appreciated that Applicant was in full possession of the claimed uses or would not have been able to practice the claimed uses using nothing more than the teachings of the specification, the knowledge in the art, and routine experimentation. Thus, Applicant respectfully submits that the instant claims amply satisfy the requirements of Article 84 and Rules 43(1) and 43(2), in contrast to the position advanced by the Examining Division. Reconsideration and withdrawal of the objection are respectfully requested.

3.2.2. *Auxiliary Requests II to VI*

Auxiliary Requests II to VI recite "eculizumab" in all of the claims, thus, rendering the rejection moot.

3.3. Result-to-be achieved

3.3.1. At page 4 of the Office Action, claims 3 and 10 were objected to as allegedly lacking written description. According to the Examiner, claims 3 and 10

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impermissibly attempt to define subject matter in terms of a result to be achieved allegedly in contravention of Article 84 EPC and the Guidelines for Substantive Examination, specifically Chapter III, 4.10 [hereinafter the Guidelines].

3.3.2. Applicant respectfully disagrees with the Examiner's characterization of the claims. However, solely in the interest of expediting prosecution, the present amendments to claims 3 and 10 are believed to render the objections moot.

In the event that the comments in the Summons have bearing on any of the instant claims, Applicant provides the following additional remarks.

According to the Guidelines, claims defined by results to be achieved are allowable if: (a) the invention can only be defined in such terms or cannot otherwise be defined more precisely without unduly restricting the scope of the claims and (b) the result is one which can be directly and positively verified by tests or procedures adequately specified in the description or known to the person skilled in the art and which do not require undue experimentation. Even assuming *arguendo* that the instant claims are defined by a result to be achieved, Applicant respectfully submits that the instant claims nonetheless amply satisfy these legal requirements for allowability.

With respect to the first prong of the aforementioned test, Applicant again reiterates that the instantly amended claims are drawn to novel, inventive, and fully-supported uses of an anti-C5 antibody to prevent serum complement activity breakthrough in a patient afflicted with PNH. Claim 1, for example, is drawn to an anti-C5 antibody for use in preventing serum complement activity breakthrough in a patient afflicted with PNH, wherein the anti-C5 antibody is to be administered to the patient to maintain a serum complement activity level below 20% of the normal serum complement activity.

As acknowledged by the Examining Division, the specification exemplifies the use of eculizumab in PNH patients and provides exemplary dosing regimens for maintaining serum complement activity below 20% of normal. The Examiner insists that the claims should go no further. Yet, in view of the teachings of the specification, a skilled artisan at the effective filing date of the application would have appreciated that eculizumab is not the only anti-C5 antibody that is useful to

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prevent serum complement activity breakthrough in PNH patients as claimed. Indeed the specification provides another example of an anti-C5 antibody, pexelizumab, which is useful in accordance with the claims.

Thus, the contribution of the disclosure and indeed the scope of the claimed invention is much broader than a single antibody and, accordingly, would be unduly limited if Applicant was required, as urged by the Examining Division, to limit the claims to eculizumab. As noted above, the instant claims are not without definition. In fact, the claims set forth, among other things, the structure of the therapeutic agent used, the affirmative step required for the use, the patient population to which the use is directed, the particular pathological state sought to be avoided, and a measurable, concrete physiological condition to be reached in the patients. Thus, the skilled artisan would have appreciated that like an ashtray for automatically extinguishing a cigarette based on the shape and relative dimensions of the ashtray, the instantly claimed uses "vary considerably in a manner difficult to define whilst still providing the desired effect." The Guidelines, Chapter III, 4.10. Yet the teachings of the specification and the essential features set forth in the claims provide "adequate directions to enable the reader to determine the required dimensions by routine test procedures" and demonstrate that Applicant had possession of the claimed invention at the effective filing date. *Id.*

Moreover, the specification provides and actually exemplifies methods for directly and positively determining whether serum complement activity has reached or is maintained at a level below 20% of the normal serum complement activity. For example, at page 37, line 10 to page 38, line 16 and Figure 8, the inventors disclose the experimental procedures and results of those procedures evaluating the percentage of serum complement activity in PNH patients over time. In view of this, it is abundantly clear that a skilled artisan could have readily and easily practiced the claimed uses using no more than the teachings of the specification, the knowledge in the art, and routine experimentation.

In view of the foregoing, Applicant respectfully submits that the instant claims amply satisfy the requirements of Article 84 EPC and the Guidelines cited by the Examiner. Accordingly, reconsideration and withdrawal of the objection are respectfully requested.

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3.3.3. *Auxiliary request IV*

The claims of fourth auxiliary request do not make use of features characterized by the Examining Division as "result-to-be-achieved".

3.4. The term "about"

3.4.1. At page 4, the Examining Division objected to the word "about" used in claim 10.

3.4.2. The amendments to claim 10 (now claim 12) are believed to render this objection moot.

4. PRIORITY AND NOVELTY (Articles 87 and 54 EPC)

4.1. At page 5 of the Office Action, the Examiner objected to claims 3 and 10 as allegedly being anticipated by D6. According to the Examining Division, the priority document allegedly does not disclose "dosing schedules for the maintenance of a serum concentration of antibody above 35 µg/mL...[and thus] D6 is...citable as prior art." While Applicant does not concede to any aspect of the Examiner's stated reasons for objection, claims 3 and 10 have been cancelled thereby rendering the objection moot.

4.2. The instant claims find full written description support in the priority document [see, e.g., U.S. patent application serial no. 10/771,552 at page 4, lines 16-21; page 5, lines 3-7; and page 34, lines 13-19] and thus D6 is not available as prior art with respect to these claims.

4.3. The claims of the fourth auxiliary request do not refer to the feature "effective to maintain a serum concentration of the antibody of above 35 µg/ml". Applicant thus submits that the new claims are entitled to the claimed priority and consequently D6 is not available as prior art with respect to the new claims.

5. INVENTIVE STEP (Article 56 EPC)

5.1. At pages 5 and 6 of the Office Action, claims 1 to 14 were objected to as allegedly lacking inventive step over D2 and D9, in view of D1, D3, and D4.

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According to the Examining Division, the instant claims represent nothing more than the "optimisation of a known dosing schedule for individual patients". Applicant disagrees with this characterization of the claimed invention.

5.2. As noted above, the instant claims are drawn to a novel and inventive use of an anti-C5 antibody to prevent serum complement activity breakthrough in PNH patients. The claims are based, at least in part, on the discovery by the inventors that the devastating hemolytic paroxysms experienced by PNH patients can be effectively controlled by maintaining the patients' serum complement activity at a level below 20% of normal serum complement activity. Failure to maintain serum complement activity below 20% resulted in breakthrough and resumption of the associated debilitating and life-threatening sequelae associated with PNH. As stated in the description of the instant application, this "[b]reakthrough of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with a return of serum hemolytic activity." *See* specification at page 35, lines 8 to 22.

In view of these observations, the inventors appreciated that administering a complement inhibitor (e.g., an anti-C5 antibody such as eculizumab) to a PNH patient in a manner that consistently maintains this experimentally determined low level of serum complement activity is necessary to consistently prevent breakthrough in patients afflicted with PNH. Claim 5 of the Main Request, further requires testing, subsequent to administration of the antibody, to confirm that the reduced serum complement activity has been maintained in the PNH patients. Thus, one of skill in the art at the effective filing date of the application would have immediately recognized from the specification that the claimed invention does not merely represent routine dosage optimization; rather, the claimed invention captures a new therapeutic modality and approach to PNH based on the recognition of the precise physiological levels of complement activity required to stave off PNH-related pathologies. When read through this lens, the cited references, alone or in combination, clearly fail to deprive the instant claims of inventiveness. Applicant describes each of the cited references below.

D2 is a short article related to the preliminary results of a clinical trial in which eculizumab was administered to patients with PNH. D9 is a scientific meeting abstract that, like D2, discloses some preliminary results of a clinical trial related

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to eculizumab. The authors of D9 note, for example, that eculizumab was useful in reducing the need for transfusion in patients with PNH. These references, however, are silent on the need to maintain PNH patient serum complement activity at a level below 20% of normal complement activity or testing for such activity. There is certainly no disclosure or even a suggestion of breakthrough in D2 or D9, that maintaining serum complement activity below 20% of normal could prevent breakthrough, or even of the specific applications (e.g., the specific dosage regimens) of the instantly claimed invention embodied in claims 2 and 3 of the Main Request.

D3 is a short article related to approval of orphan drug status for eculizumab in PNH treatment. D4 is a scientific manuscript relating to the use of warfarin to inhibit thrombosis in PNH patients. These references, even more so than D2 and D9, fail to disclose or suggest any specific treatment conditions for an anti-C5 antibody, let alone the need to maintain PNH patient serum complement activity at a level below 20% of normal complement activity or testing for such activity. There is certainly no disclosure or even a suggestion in D3 or D4 of breakthrough or that maintaining serum complement activity below 20% of normal could prevent breakthrough as claimed.

D10 is a review article summarizing the generation and characteristics of eculizumab as a therapeutic agent. D10 discloses that eculizumab may be therapeutically beneficial in conditions such as, but not limited to, myocardial infarction, ischemia-reperfusion injury, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). *See* D10 at, e.g., page 1019. The reference further notes that Alexion (the instant Applicant) was at the time of the reference's publication engaged in clinical trials evaluating the use of eculizumab in, e.g., RA, SLE, psoriasis, and lupus nephritis. The Examiner cites to D10 for the purpose of establishing the activity of a single dose of eculizumab in patients with SLE. However, D10 does not disclose or suggest the use of an anti-C5 antibody to prevent serum complement activity breakthrough in patients afflicted with PNH. In fact, D10 does not relate to PNH at all. There is certainly no recognition by the authors of D10 that maintaining a level of serum complement activity below 20% of normal is necessary to consistently prevent breakthrough in patients afflicted with PNH.

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D1 published in December 2004 as reflected on the face page of the journal issue in which the article appeared (attached). As a December 2004 publication date is well after the priority date of the instant application (February 3, 2004), D1 is simply unavailable as prior art against the instant claims.

Thus, none of the cited references disclose or suggest maintaining PNH patient serum complement activity at a level below 20% of normal complement activity.

In the event that the foregoing arguments are not accepted by the Examining Division, Applicant submits the following arguments relating to specific dependent claims and the Auxiliary Requests I to III for consideration. Claims 2, 3, 10 and 11 (and all claims of Auxiliary Request 3) recite that eculizumab is to be administered to the patient at least once every 12 days at a dose of at least 900 mg. The fact that the PNH symptoms of all of the treated patients in the study of the Applicant were prevented by using this dosing regimen was surprising. The skilled artisan would not have foreseen this result based on any combination of the cited art. Nor would the skilled artisan have had any reason to modify the dosage regimen of 900mg of eculizumab every 14 days that was recited in D2.

Furthermore, claims 5 and 13 (and all claims of Auxiliary Request 1) recite that the patient's serum complement activity is to be tested to determine if the serum complement activity is maintained at a level below 20% of normal serum complement activity. Testing the patient's serum complement activity is not recited in any of the art cited by the Examining Division. At the priority date, one of ordinary skill in the art would not have been motivated to test the patient's serum complement activity because there was no recognition in the art that maintaining a level of serum complement activity below 20% of normal is necessary to consistently prevent breakthrough in patients afflicted with PNH.

In view of all of the foregoing arguments, Applicant respectfully submits that the instant claims represent a therapeutically useful, novel, and inventive approach over the cited prior art. Accordingly, reconsideration and withdrawal of the objection are respectfully requested.

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5.3. *Auxiliary request IV*

The arguments presented above apply *mutatis mutandis* to the fourth auxiliary request.

It is moreover emphasized with regard to the fourth auxiliary request that the issue in assessing inventive step is not whether the skilled person could have arrived at the invention by adapting or modifying the closest prior art, but whether he would have done so because the prior art incited him to do so in the hope of solving the objective technical problem or in expectation of some improvement or advantage.

D2 fails to disclose the administration of 900 mg eculizumab once every 12 days. It is concluded in D2 that the used dosage regimen of 900 mg eculizumab once every 14 days is effective in the treatment of PNH.

As regards D10, we note that this disclosure does not all refer to a specific dosage regimen of eculizumab in the treatment of PNH. It is emphasized that D10 refers to the treatment of SLE (systemic lupus erythematosus). The dose used for the treatment of SLE cannot provide any information about the treatment of PNH. Moreover, a document referring to a different medical indication does not qualify as secondary prior art. If it were nevertheless to be considered, we submit that the skilled person does not find any suggestion therein for a fine-tuning of dosage in the treatment of PNH.

Also the remainder of the cited prior art does not provide any incentive to administer 900 mg eculizumab every 12 days.

The inventors have thus surprisingly found that - upon the initial dosage regimen step (i) and (ii) – as step (iii) 900 mg eculizumab every 12 days is required in order to effectively and consistently block serum hemolytic activity; cf. page 35, lines 5-22. In more detail, 900 mg eculizumab every 14 days is not sufficient to block complement break-through in all patients. The fact that 900 mg eculizumab every 14 days may suffice in most patients to block complement break-through cannot be detrimental to inventive step, because only the dosage regimen according to the invention ensures that all patients are successfully treated.

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This dosage regimen is technically advantage as compared to the use of 900 mg eculizumab every 14 days (D2). As disclosed at page 35, lines 5-22 of the application as filed 900 mg eculizumab every 14 days are not sufficient in order to effectively and consistently block serum hemolytic activity in PNH patients.

Taken together only the result of presented in the examples of the present application would led the skilled person to a dosage of 900 mg eculizumab every 12 days. It is therefore submitted that the claims of the fourth auxiliary request are inventive.

6. FURTHER REQUESTS

With the above amendments and explanations, it is submitted that the new main request meets the requirements as set forth in the Summons. In case the Examining Division should come to the conclusion that the main request is not patentable, consideration of the first auxiliary request, the second auxiliary request, third auxiliary request and the fourth auxiliary request (in that order) is requested.

If the Examining Division shares this view, then it would seem that it were no longer necessary to hold oral proceedings. In this case it is requested that oral proceedings be cancelled and the proceedings be continued in writing. Otherwise, our request for oral proceedings according to Article 116(1) EPC is maintained. In the latter case it is requested that filing of at least one further request during oral proceedings is allowed. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the telephone.

Dr. Roger Abseher
European Patent Attorney

Enclosure:
New claim sets

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The examination is being carried out on the **following application documents**

Description, Pages

1-55 as originally filed

Sequence listings, SEQ ID NO

1-10 as originally filed

Claims, Numbers

1-21 as originally filed

Drawings, Sheets

1/4-4/4 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01)

D2 HILLMEN P ET AL: "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)",
BRITISH JOURNAL OF HAEMATOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB,

vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758,
ISSN: 0007-1048

D3 HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA",
NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US,
vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948,
ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688

D4 HARDING J: "Eculizumab",
DRUGS OF THE FUTURE, PROUS SCIENCE, ES,
vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000,
ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330

D5 US 2005/221382 A1 (ROTHER RUSSELL P [US]) 6 October 2005 (2005-10-06)

D6 HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria",
CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US,
vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426,
ISSN: 1543-0790

For what concerns the most relevant passages of the above-mentioned documents , please see citations in the European Search Report, unless otherwise stated.

1. Novelty

The present application formally meets the requirements of Article 54(1) and (2) EPC because the subject-matter of the claims is not exactly disclosed by the available prior art documents.

2. Inventive step

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of the whole set of claims does not involve an inventive step within the meaning of Article 56 EPC.

Document D1 may me considered as the closest prior art to the subject-matter of the present application and discloses the use of any compound which binds or otherwise blocks the generation and/or activity of one or more complement component in the treatment of paroxysmal nocturnal hemoglubinuria. In particular, anti-C5 antibodies are described by D1: eculizumanb and pexelizumab.

D1 also discloses the dosage regimen for the described treatment: an aqueous solution comprising eculizumab is administered to the patients in a dosage of 600 mg weekly for four weeks, followed by a 900 mg eculizumab one week after, and 900 mg eculizuman bi-weekly till the end of the treatment. The dosage regimen indicated by D1 corresponds exactly to the one claimed in present claims 15 and 19, both referring back to a single unit dosage form of the ant-C5 antibody or C5-binding fragment thereof.

It should be noted by the applicant that the dosage regimen specified in the application and disclosed by D1 is to be considered as the conventionally used one since it is disclosed by many other prior art documents (see for example D2-D4).

The difference between the claimed subject- matter and the disclosure of D1 is therefore nly represented by the indication that the claimed pharmaceutical composition comprises a single unit dosage form.

As a first remark, it should be noted that the present application does not indicated any advantage linked to the formulation of the claimed compounds in a single unit dosage form compared to any other forms disclosed by the prior art.

Moreover, D5 already teaches that aqueous formulations of the disclosed compounds, although used for the treatment of a different disease, may be presented in unit dosage forms and prepared according to conventional techniques well known in the pharmaceutical industry.

Thus, in the absence of any surprising or unexpected technical effect due to the formulation of the claimed compounds in a single unit dosage from, the skilled person would have come to the claimed invention without any exercise of an inventive activity.

The attention of the applicant is also drawn to the fact that a single unit dosage form of the mentioned compounds in an amount of 300 mg, would obviously be the most convenient one since for administering 600 mg of the active, two dosage forms and for administering the 900 mg of the active three of them can be used without any wast of active agent.

3. Further procedure of the application

It is not at present apparent which part of the application could serve as a basis for a new, allowable claim. Should the applicant nevertheless regard some particular matter as patentable, an independent claim should be filed taking account of Rule 43 (1) EPC. The applicant should also indicate how the subject- matter of the new claim differs from the state of the art and the significance thereof.

The attention of the applicant is drawn to the fact that the application may not be amended in such a way that it contains subject- matter which extends beyond the content of the application as filed (Article 123(2) EPC).

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

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16. Nov. 2012

EP 11 00 1632.6 - 2123
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/1

München, November 15, 2012
WA/ROA/BUK

This is in reply to the Communication pursuant to Art. 94(3) EPC dated July 10, 2012:

1 AMENDMENTS TO THE CLAIMS

1.1. New claims 1 to 3 correspond to previous claims 1 to 3.

1.2. New claim 4 is based on previous claim 4 and has been made into a dependent claim referring back to claims 1 to 3. Said reformulation is supported by previous claim 4.

1.3. New claim 5 corresponds to previous claim 5.

1.4. New claim 6 is based on previous claim 6. The claim now only refers to an anti-C5 antibody.

1.5. New claims 7 to 19 correspond to previous claims 7 to 19.

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1.6. New claim 20 is based on previous claim 20 and now properly refers back to the subject-matter of claims 1 to 3.

1.7. New claims 21 to 23 correspond to previous claims 21 to 23.

2 INADMISSIBLE BROADENING (ARTICLE 123(2) EPC)

2.1. Claims 1 to 3

2.1.1. In sections 1.1 of the Communication, the Examining Division objects to the feature that the patient has been vaccinated against Neisseria meningitides or the claimed pharmaceutical composition is administered to patients in conjunction with a vaccine against Neisseria meningitides in claims 1 to 3. According to the Examining Division, support for these claims is derived from the working examples and "a feature taken from an example cannot be generalised to the general teachings of the invention without infringing Article 123(2) EPC."

2.1.2. Applicant cannot subscribe to the assessment of the Examining Division and submits the following. The new claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The anti-C5 antibody inhibits cleavage of complement component C5 into fragments C5a and C5b. The claims also require that the patients be vaccinated against *Neisseria meningitides* (see claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (see claims 2 and 3).

2.1.3. The compositions as recited in the claims are supported at, e.g., page 5, lines 3 to 9 of the specification (WO 2007/106585). The use of such compositions in the treatment of patients with hemolytic diseases is disclosed at, e.g., page 8, lines 19-21 and page 22, lines 7-17. Thus, the question is whether the specification provides support for the introduction of the feature of vaccination

against *Neisseria meningitides* as mentioned in the example section which is alleged to constitute an undue generalization. As indicated in detail below, Applicant submits that the specification clearly supports the presently claimed subject matter.

2.1.4. With regard to the objective of Article 123(2) EPC and the question of allowable generalization, a considerable body of case law has developed and has partially been manifested in the Guidelines for Examination in the European Patent Office (hereinafter referred to as "Guidelines") to ensure that the claims of a European patent application are not amended in such a way that they contain subject-matter which extends beyond the content of the application as filed.
In relation to the compliance of a correction under Rule 43(2) EPC to Art. 123(2) EPC it was ruled that if it

> "expresses what a skilled person, using common general knowledge, would already derive on the date of filing from the parts of a European patent application, seen as a whole" (G11/91, reasons for the decision, paragraph 4)

a correction would not infringe Art. 123(2) EPC.

According to the Guidelines at Part H, Chapter IV, 2.2, an amendment fails to comply with Article 123(2) EPC if it

> "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." (Guidelines, Part H, Chapter IV, 2.2)

The Guidelines further clarify that implicit subject matter is

> "no more than the clear and unambiguous consequence of what is explicitly mentioned in the application as filed." (Guidelines, Part H, Chapter IV, 2.3)

2.1.5. As evident from the above, compliance with Article 123(2) EPC does not require *ipsis verbis* support in the description but extends to subject-matter being

explicitly disclosed in other terms or implicitly disclosed. For example, in T 0878/09 (reasons for the decision, paragraph 29) the Board stated that the

> "determination of whether or not the provisions of Article 123(2) EPC are fulfilled requires the determination of 'subject matter'....[and s]ince the same subject-matter may be described by different words – for example the same glass may be described as either 'half full' or 'half empty' – a difference in wording does not per se necessarily establish that the subject-matter defined by the words is different."

2.1.6. It is further not categorically the case that a specific feature taken from the specification cannot be generalized to a more general invention without infringing Article 123(2) EPC. Rather, the Board has held that Article 123(2) is not satisfied in cases where, for example, the as-filed specification: (i) describes a specific embodiment of a claimed invention and (ii) the general applicability of that embodiment would not have been evident to a person of skill in the art reading the specification (see T157/90). In the latter decision, the claims related to a calcitonin polypeptide comprising an additional amino acid at the C-terminus. The subject application taught that the additional amino acid (+1 amino acid) was required for the successful amidation of a proline residue preceding the additional amino acid. Amidation of the proline was "a feature required for biological activity" of the calcitonin polypeptide. However, the detailed description of the invention described only one embodiment: that the glycine residue (+1) is in vivo required during processing events for the amidation of the adjacent carboxyl terminal amino acids. The Board concluded that the specification, even when viewed in the context of the knowledge in the art at the effective filing date of the application, taught, if anything, that glycine is the amino acid necessary for the enzymatically processing of the precursor to arrive at the necessary amidation of the proline (see Reasons for Decision, paragraph 2.11). In fact, there was evidence of record that even glycine was not actually required for processing of calcitonin. Thus, according to the Board, disclosure of the sole embodiment glycine, without more, would not have been enough for a skilled artisan to recognize that any amino acid could be used as an amidation signal in the processing of calcitonin.

2.1.7. In a further decision T17/86 (confirmed by T284/94 and T470/05) concerning the question whether a technical feature claimed separately from the disclosed

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combination with others may be introduced into a claim without contravening Art. 123(2) EPC it was ruled that this is allowable, if it is

> "evident beyond any doubt to a skilled person reading the original description that this isolated feature on its own enables the object in view to be achieved." (T1786, reasons for the decision, paragraph 3)

and with reference to a claim for a device that it should

> "be ascertained whether the application as filed indisputably disclosed all the technical features of the device covered by the amended claim and whether it is clear beyond any doubt to a skilled person that the device can function independently of any other added element to achieve the object of the invention as now claimed." (T1786, reasons for the decision, paragraph 3)

2.1.8. The existing case law regarding this issue has been integrated into the Guidelines in the form of three requirements that need to be met (see Part H, Chapter V, 3.2.1):

a) the feature is not related or inextricably linked to the other features of that embodiment, and

b) the removal of the omitted features from the embodiment passes the essentiality test:

   i. the feature was not explained as essential in the disclosure;

   ii. the feature is not, as such, indispensible for the function of the invention in the light of the technical problem the invention serves to solve; and

   iii. the replacement or removal requires no real modification of other features to compensate for the change,

   and

c) the overall disclosure justifies the generalizing isolation of the feature and its introduction into the claim.

2.1.9. In decision T190/99, the Board has also stated that

> "the skilled person when considering a claim should rule out interpretations which are illogical or which do not make technical sense. He should try, with synthetical propensity i.e. building up rather than tearing down, to arrive at an interpretation of the claim which is technically sensible and takes into account the whole disclosure of the

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> patent. The patent must be construed by a mind willing to understand not a mind desirous of misunderstanding." (T 0190/99, Reasons for the Decisio, paragraph 2.4).

2.1.10. Out of the foregoing law, several intertwined principles emerge. First, neither the Guidelines nor the case law articulates an Article 123(2) EPC analysis that is inflexible or narrow; instead, the analysis considers the context in which the amended claims sit, i.e., the teachings of the patent application disclosure as a whole and what those teachings mean implicitly within the milieu of the common scientific knowledge in the art. Second, the analysis must be conducted from the viewpoint of a skilled person who is interested in, and "willing to understand", the disclosure and what it teaches him/her.

2.1.11. Under this analysis, it becomes evident that a skilled person reading the instant disclosure would have immediately recognized that the specification clearly defined not only a population of patients vaccinated against meningitis, but therapeutic compositions used in conjunction with such vaccination.

2.1.12. Specifically, both the original claims and the specification itself provide a supportive context for the instant claims. The skilled person would have readily appreciated that the primary aim of the specification is providing therapeutic solutions to the problem of hemolytic diseases such as paroxysmal nocturnal hemoglobinuria (PNH). For example, in its *Background* section starting at page 1, the application begins with a detailed discussion of the problem of PNH. The skilled person was clearly aware, as were the inventors of the invention covered by the pending application, of the need in the art at the filing date of the application for "therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH" (see *Background* section). Likewise, the skilled person would have immediately recognized that the numerous treatment modalities set forth in, and exemplified by, the application – particularly using compositions comprising an antagonist anti-C5 antibody – are an answer to this need.

2.1.13. Not only does the specification provide this array of therapeutic approaches, but the skilled artisan would have also appreciated that a number of specific patient populations are identified by the inventors as being amenable to such

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approaches. These patient populations include, e.g., patients having hemoglobin levels less than 14 g/dL if a man, or less than 12 g/dL if a woman (see specification at, e.g., page 3, lines 12 to 14). Other patient populations described are, e.g., those who are anemic or those who remain anemic after treatment with a composition comprising an antagonist anti-C5 antibody. (see specification at, e.g., page 3, lines 3 to 13). Indeed, the general class of patients with PNH is defined by the specification as having "an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A" (see page 1, first sentence).

2.1.14. Yet additional subpopulations of hemolytic disease-suffering patients are also set forth in the application. For example, in the Examples (see page 27 et seqq.), which exemplify the successful treatment of patients afflicted with PNH, the patients so treated are defined by a number of clinical attributes: 18 years of age or older, diagnosed as having PNH with a type III erythrocyte population of greater than or equal to 10%, and having had at least four transfusions in the 12 months prior to treatment with the antagonist anti-C5 antibody eculizumab.

2.1.15. The skilled person would have also immediately appreciated another clinical feature of these patients: namely that "all patients were vaccinated against *Neisseria meningitides*" (see page 27, line 15). A mind willing to understand would understand that the inventors believed that all patients being treated with a complement inhibitor should be vaccinated and, thus, such action was not meant to be limited to a single example given in the application. In fact, there is no example presented in which a patient was not vaccinated. Also, there is no language in the exemplification at page 27 to indicate that use of the vaccine was meant to be limited to that example. As such, and referring to the criteria developed in the Guidelines as regards generalization, neither the general part of the application nor the specific example describes the feature to be inextricably linked to or related to the other features of said example, i.e. requirement a) as laid out in the Guidelines is met (see section 2.1.8, *supra*). Following therefrom, the feature is neither explained as essential or as indispensable for the function of the invention nor does the replacement or removal require a real modification of other features to compensate for the

change. Thus, also requirement b) as laid out in the Guidelines is met (see section 2.1.8, *supra*). Moreover, also requirement c) as laid out in the Guidelines is met (see section 2.1.8, *supra*), because as explained herein above, the invention relates to the treatment of patients afflicted with hemolytic disease and vaccination is a further measure to ensure treatment, as increased frequency of neisserial infections were observed in said patients. As said increased frequency is only related to the disease and not other parameters a generalizing isolation is justified, moreover so, as the specification is silent as to any limitation to specific embodiments. At least for these reasons, the introduction of the objected feature represents no undue generalization.

2.1.16. Thus, as elsewhere in the specification, the Examples clearly and unambiguously define characteristics of a novel patient population that the inventors believed would benefit, and did benefit, from treatment with an antagonist anti-C5 antibody. Additional patient population characteristics are presently recited in the instant claims, e.g., see claims 20, 22, and 23 of the enclosed claim set. And, as a practical matter, other than an objection for being based on a rejected base claim, the Office does not appear to contest the absolute support for these defined patient populations. Applicant's independent claims simply expressly articulate characteristics of a fully-defined and -described patient population set forth in the specification.

2.1.17. For the avoidance of doubt, this is not a case where an attempt has been made to extrapolate a general feature from a *specific embodiment* set forth in the application, as alleged by the Examiner. That is, unlike the subject claim in T157/90, for example, in which one embodiment alone, glycine, was provided as support for a broader genus of any additional amino acid, Applicant in this case points to a feature (a class of patients defined by their vaccination status) specifically recited in the application as the supportive basis for the same feature of the same scope recited in the claims. In other words, Applicant has merely taken a fully supported and manifestly clear limitation already existing *in haec verba* in the application and incorporated that limitation into the general invention now claimed. Applicant respectfully submits that a skilled person at the effective filing date of the application would have found such claims directly and unambiguously derivable from that previously presented by the application,

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which, as stated above, is the requirement for sufficient support according to the Guidelines.

2.1.18. Accordingly, it is respectfully requested that the objection to claims 1 to 3 is withdrawn.

2.2. Claim 4

2.2.1. Claim 4 is objected to as allegedly being in violation of Article 123(2) EPC because the expression used in the claim "altered constant region that exhibits decreased effector function relative to the corresponding native constant region" is allegedly not equivalent to the description, in which "the decreased effector function is said to be relative to an anti-CDCP1 antibody with a native constant region." Applicant traverses.

2.2.2. As noted above, the Technical Board of Appeal stated that, when reading a specification, interpretations which are illogical or which do not make technical sense should be ruled out. The reader should try, with synthetical propensity i.e. building up rather than tearing down, to arrive at an interpretation of the claim which is technically sensible and takes into account the whole disclosure of the patent. The patent must be construed by a mind willing to understand the invention (see T0190/99, loc. cit.). Applicant respectfully submits that the skilled person would immediately appreciate that the recitation of an "anti-CDCP1 antibody" is a simple typographical error in place of which an "anti-C5 antibody" was intended. Whereas the application recites "an anti-C5 antibody" exhaustively, nowhere else does the CDCP1 antibody appear in the document. The skilled person would immediately and directly understand that the "antibody that binds C5...[that] comprises an altered constant region....that exhibits decreased effector function" would be relative to the same antibody having a native constant region. This understanding is further underlined by the general knowledge in the art of antibody engineering[1] and the skilled person's

[1] *See*, e.g., WO 2007/084321 at page 42, second full paragraph stating "[a] native sequence Fc or constant region comprises an amino acid sequence identical to the amino acid sequence of a Fc or constant chain region found in nature. A variant or altered Fc or constant region comprises an amino acid sequence which differs from that of a native sequence heavy chain region by virtue of at least one amino acid modification, insertion, or deletion, for example. In certain embodiments, the variant or altered constant region has at least one amino acid substitution, insertion, and/or deletion, compared to a native sequence constant region or to the constant region of a parent polypeptide".

understanding of the structural features of eculizumab, for example, which was known to comprise "a G2/G4 [constant region] in place of the G1 domain" (see specification at page 5, lines 16 to 18;see also the package insert for Soliris®, the trade name under which eculizumab is sold, at paragraph 11 (a copy of the package insert is submitted herewith as **Exhibit A**)). Applicant also provides Mueller et al. (1997) *Mol Immunol* 34(6):441-452 as **Exhibit B**, which describes a G2/G4 Fc portion of an antibody as lacking complement activating activity (see, e.g., page 446, column 2 and Fig. 9).

2.2.3. Thus, claim 4 does not result in the skilled person being presented with information which is not directly and unambiguously derivable from that previously presented by the application, particularly when account is taken of matter which is implicit to said skilled person and the passage is read in the context of the application as a whole. Accordingly, reconsideration and withdrawal of the objection to claim 4 are respectfully requested.

2.3. Claim 6

2.3.1. Claim 6 was objected to as allegedly violating Article 123(2) EPC because the "dosage regimen given on page 22, lines 13-17 only refers to administration of the antibody and not to the one of the C5-binding fragment as claimed."

2.3.2. While not conceding to any aspect of the Examining Division's stated reasons for objection, previous claim 6 has been amended. As amended, the claim specifies that an anti-C5 antibody is to be administered to the patient under the specific dosage regimen recited. It is submitted that such an amendment renders the objection moot and therefore respectfully requests reconsideration and withdrawal of the objection to claim 6.

2.4. Claims 1 to 23

2.4.1. The Examiner notes with regard to the claims that "it is not possible to combine features taken from different independent embodiments or from the examples without violating the requirements of Article 123(2) EPC".

2.4.2. As noted above, neither the Guidelines nor the case law articulates an Article 123(2) EPC analysis that is as inflexible as that set forth in the Office Action. For example, in decision T1241/03 the Board denied that a claim "need[s] to have a literal basis in a single passage of the application as originally filed" to comport with Article 123(2) EPC (see T1241/03, Reasons for the Decision, paragraph 7). In T1241/03, the claims at issue were drawn to stable, aqueous liquid pharmaceutical formulations comprising human growth hormone (hGH) and a buffer providing pH in the range of 5.5 to 7, 0.1 to 1% by weight of a non-ionic surfactant and a neutral salt (T1241/03, Reasons for the Decision, paragraph 4). The claims were challenged as violating Article 123(2) because "the application as originally filed did not explicitly disclose the claimed formulation" in a single passage (T1241/03, Reasons for the Decision, paragraph 5). The Board rejected this challenge noting, among other things, that a general description of the claimed formulations and methods for producing stable formulations of hGH "by mixing it with a non-ionic surfactant in the range of 0.1 to 5% (w/v)" were expressly disclosed in the specification, albeit in separate passages (T1241/03, Reasons for the Decision, paragraph 6). The Board also noted that the working examples of the specification exemplified the generation and characterization of a stable formulation embraced by the claims. In view of this, the subject claims did "not refer to a 'patchwork' of parameters disclosed in non-connected parts of the description, nor [had] specific values been isolated from examples in a non-allowable way" (T1241/03, Reasons for the Decision, paragraph 7). Quite the contrary, the Board stated that considering the general disclosure of the application as filed, the reference in a claim to a combination of compounds in specific concentrations, explicitly disclosed in different passages of the application, is not considered to be an amendment of the patent which extends beyond the content of the application as originally filed.

2.4.3. Moreover, in another decision the Technical Board of Appeal has cautioned against Article 123(2) EPC determinations based on overly narrow readings of isolated passages in an as-filed specification. According to the Board, the question whether or not the instantly claimed subject matter is disclosed in the application as originally filed must take account of the whole content thereof and

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cannot solely be based on isolated parts of the description (see T823/96, Reasons for the Decision, paragraph 4).

2.4.4. In view of these legal principles, Applicant respectfully submits that the instant claims sufficiently satisfy the requirements of Article 123(2) EPC. The instant claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The anti-C5 antibody inhibits cleavage of complement component C5 into fragments C5a and C5b. The claims also require that the patients be vaccinated against *Neisseria meningitides* (e.g., claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (e.g., claims 2 and 3).

2.4.5. The claimed compositions (the single unit dosage forms) find full support, e.g., in the specification at page 5, lines 3 to 9. Further unequivocal support for administering such compositions to patients with hemolytic diseases is set forth in the same passage as well as at page 8, lines 19 to 21 and page 22, lines 7 to 17. And as noted above, support – particularly when viewed in the context of the general knowledge in the art and the entire teachings of the specification – for the treatment of a population of patients vaccinated against *Neisseria meningitides* is provided at page 27, line 15. In fact, the claims define characteristics of the patient population actually treated by the inventors.

2.4.6. Therefore, contrary to the position taken by the Office Action, it would have been clear to a skilled person that the instant specification sufficiently described the treatment of vaccinated patients afflicted with a hemolytic disease using the compositions as claimed. Like the claim upheld in T1241/03, it is clear that claims 1 to 3 do not refer to a 'patchwork' of parameters disclosed in non-connected parts of the description, nor have specific values been isolated from examples in a non-allowable way. Rather the specification provides literal support for each element of the claimed uses and instructive and supportive language that unambiguously tethers these elements together. Thus, in the words of the Board of Appeal, "considering the general disclosure of the application as filed, the reference in a claim to a combination of compounds ...

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explicitly disclosed in different passages of the application, [should not be] considered...an amendment of the [claims] which extends beyond the content of the application as originally filed." Accordingly, as instant claims 1 to 3 (and all claims dependent therefrom) are directly and unambiguously derivable from the specification as filed, reconsideration and withdrawal of the rejection under Article 123(2) EPC are respectfully requested.

2.5. Dependent claims

2.5.1. Lastly, the Office Action also objects to all dependent claims under Article 123(2) as being based on independent claims allegedly in violation of the provision.

2.5.2. Applicant respectfully submits that for the reasons of record and those provided herein, the instant independent claims, and all claims dependent therefrom, satisfy the requirements of Article 123(2) EPC. Accordingly, reconsideration and withdrawal of this objection are respectfully requested.

3 UNITY (ARTICLE 82 EPC)

3.1. The Examining Division insinuates that in view of the previous line of arguments relating to the presence of three solutions to the posed problems the claims could be seen to lack unity.

3.2. As noted above, the instant claims are drawn to pharmaceutical compositions for use in treating patients afflicted with a hemolytic disease. The novel compositions comprise a single unit dosage form of an anti-C5 antibody, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody. The claims also require that the patients be vaccinated against *Neisseria meningitides* (e.g., claim 1), or that the composition be used in conjunction with a meningitis vaccine for treating the patient (e.g., claims 2 and 3).

3.3. Applicant respectfully submits that the Examining Division has at least constructively acknowledged that such claims – including the linking concepts of

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the particular compositions and the use in conjunction with a meningitis vaccine or in vaccinated patients – are novel over the prior art. The instant claims are also inventive over that art for the reasons of record and those that follow.

4 INVENTIVE STEP (ARTICLE 56 EPC)

4.1. In section 3 of the Communication, the Examining Division maintains its inventive step objection over D1 in combination with D5 raised in the ESOP which follows the problem-solution-approach. According to the Examining Division, Applicant's prior arguments in favor of inventiveness were not "appropriate in that they [allegedly] focus on the differences between D1 or D5 and the claimed invention....the complementary teachings of D1 and D5 have been combined for assessing the inventiveness of the invention."

4.2. Applicant cannot follow the Examining Division's assessment. The claimed invention is based, at least in part, upon Applicant's discovery that during an early clinical trial evaluating eculizumab as a safe and effective therapy, a patient undergoing complement suppressive therapy developed a meningitis infection. In view of this previously unrecognized problem, and to further safeguard the well-being of PNH patients in need of eculizumab therapy, Applicant affirmatively chose to treat PNH patients with an anti-C5 antibody only after the patients had been vaccinated against *Neisseria meningitides*, or where the antibody was used in conjunction with such a vaccination. The instant claims embrace this inventive concept. Claim 1, for example, is drawn to the use of anti-C5 antibody-containing composition for treating PNH patients who have been vaccinated against *Neisseria*. Claims 2 and 3 are drawn to embodiments in which the anti-C5 antibody-containing composition is to be therapeutically administered to PNH patients in conjunction with vaccination.

4.3. Whether alone, or in combination, the cited references fail to disclose or suggest such uses.

4.4. D5, which *per se* fails to disclose the instantly claimed pharmaceutical composition unit dosage forms, does not relate to hemolytic disease at all, but

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rather methods for treating, among other things, rheumatoid arthritis. And although the methods of D5 can include the use of an anti-C5 antibody, there is no disclosure or even a suggestion in D5 of using the claimed compositions in patients that have been vaccinated against *Neisseria meningitides*. Therefore, D5 does not deprive the instant claims of inventiveness. However, the Communication appears to assert that there would have been a reason to combine the teachings of D5 with that of D1 to thereby arrive at the instantly claimed uses. Applicant submits that this is not the case. In fact, absent the teachings of the specification, there would have been no reason at all for a skilled artisan to practice the uses now claimed.

4.5. D1 teaches, among other things, methods for treating PNH using an anti-C5 antibody. As acknowledged by the Examining Division, however, the reference does not disclose pharmaceutical compositions comprising a single unit dosage form of 300 mg of an anti-C5 antibody. D1 does make one reference to meningitis at paragraph [0041], which characterizes the general knowledge in the art at the time as being that "patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone". However, this passage alone would not have provided the requisite inertia for a skilled person to, first, make a composition neither described nor suggested in D1 or D5, and then use such a composition in conjunction with a meningitis vaccine or in vaccinated patients. Only with the guidance provided by the inventors in the instant specification would there have been a reason for a skilled person at the effective filing date of the application to arrive at the instantly claimed uses.

5 REQUESTS

With the above amendments and explanations it is submitted that the Applicant has met the requirements as set forth in the Communication. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

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If, however, the Examining Division does not agree with the above, it is requested that either a further Communication pursuant to Art. 94(3) EPC and Rule 71(2) EPC or a summons to attend oral proceedings according to Art. 116(1) EPC be issued. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure
New claims 1 to 23
Exhibit A and B

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Date: 06-09-2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888

Applicant/Proprietor: Alexion Pharmaceuticals, Inc.

Acknowledgment of receipt of observations by third parties (Article 115 EPC)

Receipt of your letter dated 02.09.2019 is hereby acknowledged.

Under Article 115 EPC you will not be a party to the proceedings before the European Patent Office.

☐ In your letter the following documents are mentioned which were not enclosed, and which are not available in the EPO:

☐ The third party observations have not been filed in an official language of the EPO (R. 114(1) EPC).

You are requested to file copy(ies) and/or translation(s) in one of the official EPO languages within **two months** of notification of this communication if they are to be taken into account.

For the Examining Division

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Date
09-09-2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication pursuant to Rule 114(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115 EPC).

Under Rule 114(2) EPC you may comment on the observations.

For the Examining Division

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Page 1015

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Our ref: SK/6930 Your ref: **EP 3 167 888** Wroclaw, September 4th, 2019

Re: **Third Party Observations in respect of EP 3 167 888**

THIRD PARTY OBSERVATIONS

European Patent:	Application Number: EP 16195355.9
	Publication Number: EP 3 167 888
Applicant:	Alexion Pharmaceuticals, Inc.
Title:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Dear Sirs,

We enclose the Third Party Observations in respect of EP 3 167 888 for attention of Examining Division.

Your faithfully

Rafał Witek
Patent Attorney

Enclosures:

1. Third Party Observations in respect of EP 3 167 888
2. EP1558648_Request for correction
3. EP1558648_Original filing (WO 2004/035607 A2)
4. EP1558648_Vink declaration
5. EP2581113_Request for correction
6. EP2581113_Application as filed

4th September, 2019

EP Appl. No. 16 195 355.9 – 1466 (EP 3 167 888)
In the name of Alexion Pharmaceuticals, Inc.

Third Party Observations

We would like to respectfully bring the following Third Party Observations to the Examining Division's attention:

I. The amended claims submitted by the Applicant as Main Request contravene Articles 123(2) and 76(1) EPC

(1) In response to the Examining Division's Communication in preparation of oral proceedings, dated May 7, 2018, the Applicant argued in submissions dated August 15, 2018 and July 5, 2019, that the skilled person would have immediately recognized that the Applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he/she would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

(2) The Applicant pointed to Enlarged Board of Appeal decisions **G 3/89** and **G 11/91** to support his allegations. We note that the Applicant's arguments are based on a flawed interpretation of the cited case law.

(3) A correction under Rule 139 EPC is of a strictly declaratory nature. Two requirements arise for a correction under Rule 139 to be allowable, i.e., not to contravene Article 123(2) EPC and/or Article 76(1) EPC. First, the presence of an error must be obvious. Second, it has to be *"immediately evident [for a skilled person] that nothing else would have been intended than what is offered as the correction"* (Rule 139 EPC, second sentence). If there is any doubt, including even slight ambivalence, that nothing else would have been intended than what is offered as the correction (i.e., the correct sequence of eculizumab), a correction cannot be allowed (see also **J 16/13**, points 8 to 10 of the reasons).

(4) The Proprietor has not met these requirements as they have not shown that it would be immediately evident that the leader sequence of SEQ ID NOs: 3 and 4 would precisely consist of residues 1-22 of SEQ ID NOs: 3 and 4 and nothing else.

(5) The Proprietor alleges in this regard (par. bridging p. 2/3 of the submission dated August 15, 2018) that the skilled person would know from his common general knowledge that the *"framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat et al."* (Exhibit E filed with the Andrien Declaration on July 10, 2017). Notably, the table

of contents (Exhibit C filed with the Andrien Declaration on July 10, 2017) makes clear that the Applicant chose to submit only a fraction of the human kappa light chain sequences disclosed in Kabat *et al.* (according to the table of contents, human kappa light chain sequences are disclosed on pages 103 to 130 of Kabat *et al.* out of which only pages 103 to 108 have been submitted). The sequence variability among human kappa light chains must therefore be assumed to be considerably greater than what is derivable from the documents submitted by the Applicant.

(6) However, even within the limited selection presented by the Applicant, Asp lle Gln Met are at best commonly the first residues. When assessing the allowability of an amendment under Rule 139 EPC, the question is not whether human kappa light chains commonly start with any particular sequence, but whether they must start with a certain sequence and whether this would have been obvious to the skilled person. Otherwise, the skilled person would be left with uncertainty, at which specific residue of SEQ IDs NO: 3 and 4 the correct mature sequence (excluding the leader peptide) would begin. This is clearly the case here, which will we explained in more detail below.

(7) Specifically, one question is how many residues the skilled person would have subtracted from the sequence disclosed in the patent, if recognizing that said sequences comprise a leader peptide? Further, how would the skilled person arrive at one and only one possible sequence to deduct from SEQ ID NOs: 3 and 4? The Proprietor states that *"the last two residues [of the leader peptides] are predominantly Arg Cys"* (par. bridging p. 2/3 of the submission). Again, as the Examining Division will be aware, *"predominantly"* does not meet the standard required for a correction under Rule 139 EPC. Quite to the contrary, the fact that the Applicant uses the term "predominantly" already shows that there are alternatives to the correction that would be apparent to the skilled reader of the application. However, as noted above, there must not be any ambiguity for a correction to be allowable. Otherwise, the skilled person would be confronted by the correction with subject matter that is not originally disclosed.

(8) The Applicant thus failed to show that a skilled artisan would immediately recognize how to replace the incorrect sequences (i.e., what the specific sequence of the leader peptide would be), with certainty.

(9) The Applicant's representative further asserted that similar requests for corrections would have been accepted by the EPO, pointing to EP 1 558 648 and EP 2 581 113. Regarding EP 1 558 648, the relevant protein sequences as well as the CDR sequences were derived by standard sequencing procedures (Example 2, WO 2004/035607 A2, submitted herewith). The antibodies were then claimed by reference to V_L and V_H sequences or to CDR sequences which is also standard in the art. Moreover, the original application disclosed at p. 29, I. 35-36 that *"heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody"*.

(10) The Applicant of EP 2 581 113, had pointed to page 67, paragraphs [0222] and [0223] of the application as filed (submitted herewith), disclosing that the presence of a signal sequence in a VL or VH amino acid

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sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies.

(11) Both applications referred to by the Applicant therefore discuss the occurrence of leader sequences or a clear identification of the mature antibody by comparison to known peptides. As pointed out above, the current application as filed is completely silent about leader sequences and does not provide any guidance for the skilled person how to clearly identify the claimed antibody. Therefore, these cases cannot be compared to the present one.

(12) Notably, with the request for correction of an obvious error, the Applicant in the EP 1 558 648 case submitted an expert declaration of Tom Vink (attached hereto). In the declaration, the expert explains the following (section 10 of the Vink declaration):

> 10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

(13) According to the statement of the expert, V_L kappa signal peptides typically have a length of 19 to 23 AAs and end with C, A, G or E, while human V_L kappa chains begin with A, D, V, N or E. We note that the number of possible combinations appears to be even larger, when taking Kabat *et al.* into regard (see above). However, if this reasoning is applied to the sequences disclosed in EP 3 167 888 as filed originally (see below) at least one alternative cleavage site to the one suggested by Alexion would be apparent to the skilled person, namely between amino acids 19 and 20 (WLR**GA**RC). The resulting leader peptide would be 19 amino acids in length, end with a G residue and the resulting mature V_L chain would begin with an A residue. Thus, all requirements that the skilled person would allegedly be aware of (according to the Vink declaration) would be fulfilled.

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SEQ ID NO: 3 – Eculizumab V_L

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

(14) This is clear evidence that the skilled artisan would in the present case at the very least be confronted with two alternatives, when recognizing that SEQ IDs NO: 3 and 4 of the current application as filed erroneously comprise a leader peptide. Under such circumstances, the correction under Rule 139 EPC suggested by the Applicant would inevitably add information that is not originally disclosed.

(15) We lastly note that the *Kabat et al.* reference, as submitted by the Applicant, represents only a small (and potentially purposeful) selection from an amino acid sequence compendium that itself represents only a selection from the vast space of protein, or antibody sequences. It cannot provide evidence that nothing else than the sequences disclosed therein would have been thinkable sequences of signal peptides, or mature human kappa light chains for the skilled person.

(16) This is aggravated by the fact that the 5th edition of Kabat *et al.* was published in 1991, i.e., 15 years prior to the priority date of the application. In the quickly moving field of pharmacologically active antibodies, the space of known antibody sequences has significantly expanded within this period and thus, the sequence compendium referred to by the Applicant was long outdated at the priority date. The number of mature light chain sequences known in the art was certainly much larger in 2006, compared to 1991, accordingly, the number of possible options for the length and/or sequences of signal peptides was likely much larger in 2006, compared to 1991. Thus, the skilled person would have been confronted with even more uncertainty regarding the sequence and length of the signal peptide comprised in SEQ ID NOs: 3 and 4, even if recognizing that the sequences disclosed therein are immature.

(17) Thus, Kabat *et al.* is anyway not representative of the entire space of possible sequences the skilled person would have had to consider at the relevant date.

(18) In summary, the application provides no suggestion that specifically residues 1-22 of SEQ IDs NO: 3 and 4 are necessarily leader sequences, let alone that nothing else would have been intended than what the Applicant offered as correction. It can by no means be assumed that it would have been apparent to the skilled person that the sequence submitted by the Applicant is the only conceivable replacement for the sequences originally disclosed.

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(19) Therefore, the Applicant's request for correction of the description and the reference to residues 23 to 214 of SEQ ID NO:4, introduced into claim 1 of the MR contravene Articles 123(2) and 76(1) EPC.

II. The Auxiliary Requests filed on July 5, 2019

(20) With the submission dated July 5, 2019, the Applicant has submitted three Auxiliary Requests, AR1 to AR3. The Auxiliary Requests not only fail to overcome the objections raised by the Examining Division, they also introduce further violations of the EPC. This is explained in further detail below.

II.1 Auxiliary Request 1

(21) AR1 defines the claimed antibody by reference to SEQ ID NOs 2 and 4, however, the claim language has been amended such that claim 1 relates to an *"antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4"* instead of *"comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4"*.

(22) This alternative claim language is a clear violation of Articles 76(1) and 123(2) EPC. The application as filed refers to an antibody comprising a heavy chain and a light chain consisting of SEQ ID NOs: 2 and 4, respectively (cf. application as filed at p. 5, li. 30 to 33). In contrast, claim 1 of AR1 refers to an antibody which comprises the heavy and light chain sequences "shown in" SEQ ID NOs 2 and 4. The Applicant thus effectively replaced "consisting of" by "comprising", which in itself represents a violation of Articles 76(1) and 123(2) EPC, as the terms are not freely interchangeable (**T 759/10**).

(23) Thus, while the application as filed (and previous claim 1) defines the antibody in a closed manner (heavy and light chains consisting of SEQ ID NOs 2 and 4), claim 1 of AR1 encompasses antibodies that comprise the sequences "shown in" SEQ ID NOs 2 and 4, but may, due to the open claim language, also comprise further sequence elements. For example, an antibody comprising a heavy and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 elongated by a few further amino acids at the C-terminus would fall under claim 1 of AR1. The original disclosure does not teach such modified antibodies.

(24) The alleged basis provided by the Applicant in the letter dated July 5, 2019 (original application, p. 4, li. 32 and p. 44) fails to disclose the subject matter of claim 1 of AR1. The description text of the parent application as filed and of the divisional application as filed are identically worded. Page 4, line 32 of the original application teaches that *"[i]n certain embodiments, said antibody is eculizumab"* (emphasis added). This passage neither provides basis for the "comprising" language, nor for the vague "shown in" phrase of claim 1 of AR1. Page 44 of the application discloses the (partially incorrect) SEQ IDs. Taken together, these two passages indicate to the skilled person that the antibody disclosed in the application may be an antibody named "eculizumab", which consists of heavy chains and light chains, wherein the

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heavy and light chains consist of the sequences disclosed as SEQ ID NOs 2 and 4. A widening of this concept through the use of "comprising" language cannot be based on this disclosure.

(25) In addition, the introduction of the term "shown in" leads to a further violation of Articles 76(1) and 123(2) EPC. This is because different interpretations of this phrase are possible, at least some of which would introduce subject matter that lacks original disclosure. If *"the light chain amino acid sequence shown in SEQ ID NO: 4"* means "the light chain amino acid sequence of SEQ ID NO: 4", claim 1 of AR1 would refer to an antibody that is not eculizumab, and thus, an antibody for which protection is not actually sought, as explicitly stated by the Applicant (e.g., letter of July 5, 2019, p. 15, 1st full par.). The amendment would furthermore not solve the objections of the ED with regard to insufficiency of disclosure.

(26) Alternatively, the term "shown in" could be interpreted as meaning any light chain sequence(s) comprised in SEQ ID NO: 4. It may be assumed that this is the interpretation desired by the Applicant, because this would enable him to *inter alia* cover the (correct) light chain sequence of eculizumab via reference to the (incorrect) sequence disclosed in the application text (SEQ ID NO: 4). However, in this case, too, the amendment would not solve the objections of the ED with regard to insufficiency of disclosure, since a light chain amino acid sequence of SEQ ID NO: 4 would still be encompassed. Further, since SEQ ID NO: 4 encompasses at the very least two potential light chain sequences when interpreted in light of common general knowledge (cf. above), this claim interpretation would inevitably lead to the finding that claim 1 adds matter, since it now encompasses several (potential) light chain sequences, instead of one clearly defined light chain sequence.

(27) The introduction of the vague term "shown in" furthermore renders claim 1 wholly unclear. As explained above, it is not clear to the skilled person which exact sequence(s) are encompassed by claim 1 of AR1. The heavy and light chain sequences of SEQ ID NOs 2 and 4, or any heavy and light chain sequence(s) that are part of SEQ ID NOs 2 and 4, i.e., any sequence fragment that can be extracted from SEQ ID NOs 2 and 4 and that may serve as an antibody heavy, or light chain, respectively - together with further derivatives thereof comprising additional amino acids?

(28) In summary, AR1 violates at least Articles 84, 76(1) and 123(2) EPC.

II.2 Auxiliary Request 2

(29) Claim 1 of AR2 defines the claimed antibody by reference to *inter alia "residues 23 to 214 of SEQ ID NO: 4"*. As explained above, this reference to only part of SEQ ID NO: 4 is not derivable from the application as filed. Claim 1 of AR2 therefore suffers from the same deficiencies under Article 76(1) and 123(2) EPC as claim 1 of the Main Request.

II.3 Auxiliary Request 3

(30) AR3 is based on AR1, with claims 1 and 2 being reformulated to medical use claims by addition of the phrase *"for use in treating paroxysmal nocturnal hemoglobinuria"* to claims 1 and 2. Claim 2 is further

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amended by introduction of the definition of the antibody recited in claim 1. AR3 inherits the deficiencies under Article 76(1), 123(2) EPC and 84 EPC put forward above with regard to AR1.

Encl.
EP1558648_Request for correction
EP1558648_Original filing (WO 2004/035607 A2)
EP1558648_Vink declaration
EP2581113_Request for correction
EP2581113_Application as filed

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/035607 A2

(54) Title: HUMAN MONOCLONAL ANTIBODIES AGAINST CD20

(57) Abstract: Isolated human monoclonal antibodies which bind to and inhibit human CD20, and related antibody-based compositions and molecules, are disclosed. The human antibodies can be produced by a transfectoma or in a non-human transgenic animal, *e.g.*, a transgenic mouse, capable of producing multiple isotypes of human monoclonal antibodies by undergoing V-D-J recombination and isotype switching. Also disclosed are pharmaceutical compositions comprising the human antibodies, non-human transgenic animals and hybridomas which produce the human antibodies, and therapeutic and diagnostic methods for using the human antibodies.

HUMAN MONOCLONAL ANTIBODIES AGAINST CD20

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No.: 60/419163, filed on October 17, 2002, and U.S. Provisional Application No.: 60/460028, filed on April 2, 2003, both entitled HUMAN MONOCLONAL ANTIBODIES AGAINST CD20, and both of which are incorporated herein in their entirety by this reference.

BACKGROUND OF THE INVENTION

The CD20 molecule (also called human B-lymphocyte-restricted differentiation antigen or Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al.* (1989) *J. Biol. Chem.* 264(19):11282-11287; and Einfield *et al.* (1988) *EMBO J.* 7(3):711-717). CD20 is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs and is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. In particular, CD20 is expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson *et al.* (1984) *Blood* 63(6):1424-1433), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal tissues (Tedder *et al.* (1985) *J. Immunol.* 135(2):973-979).

The 85 amino acid carboxyl-terminal region of the CD20 protein is located within the cytoplasm. The length of this region contrasts with that of other B cell-specific surface structures such as IgM, IgD, and IgG heavy chains or histocompatibility antigens class II α or β chains, which have relatively short intracytoplasmic regions of 3, 3, 28, 15, and 16 amino acids, respectively (Komaromy *et al.* (1983) NAR 11:6775-6785). Of the last 61 carboxyl-terminal amino acids, 21 are acidic residues, whereas only 2 are basic, indicating that this region has a strong net negative charge. The GenBank Accession No. is NP_690605.

It is thought that CD20 might be involved in regulating an early step(s) in the activation and differentiation process of B cells (Tedder *et al.* (1986) *Eur.J. Immunol.* 16:881-887) and could function as a calcium ion channel (Tedder *et al.* (1990) *J. Cell. Biochem.* 14D:195).

Despite uncertainty about the actual function of CD20 in promoting proliferation and/or differentiation of B cells, it provides an important target for antibody mediated therapy to control or kill B cells involved in cancers and autoimmune disorders. In particular, the expression of CD20 on tumor cells, *e.g.*, NHL, makes it an important target for antibody mediated therapy to specifically target therapeutic agents

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against CD20-positive neoplastic cells. However, while the results obtained to date clearly establish CD20 as a useful target for immunotherapy, they also show that currently available murine and chimeric antibodies do not constitute ideal therapeutic agents.

Accordingly, the need exists for improved therapeutic antibodies against CD20 which are effective in preventing and/or treating a range of diseases involving cells expressing CD20.

SUMMARY OF THE INVENTION

The present invention provides improved antibody therapeutics for treating and/or preventing diseases associated with cells expressing CD20, including tumor-related diseases, and immune diseases, including autoimmune diseases. The antibodies encompassed by the invention are improved in that they are fully human and, thus, are potentially less immunogenic in patients.

As exemplified herein, the human antibodies of the invention mediate killing of B cells expressing CD20 by a variety of mechanisms. In one embodiment, human antibodies of the invention induce complement dependent cytotoxicity (CDC), *e.g.*, at least about 20% CDC mediated lysis, preferably about 30% CDC mediated lysis, and more preferably 40-50% mediated lysis in cells, such as chronic B-lymphocytic leukaemia (B-CLL) cells. In another embodiment, human antibodies of the invention induce apoptosis of cells expressing CD20. In another embodiment, human antibodies of the invention induce homotypic adhesion of cells expressing CD20. Furthermore, the human antibodies of the invention may induce antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20 in the presence of human effector cells (*e.g.*, monocytes, mononuclear cells, NK cells and PMNs). Furthermore, human antibodies of the invention may induce phagocytosis of cells expressing CD20 in the presence of macrophages. The human monoclonal antibodies of the invention may work by one or more of these mechanisms. Examples of cells which can be killed by human antibodies of the present invention include, but are not limited to, B cells expressing CD20, such as tumorigenic B cells and B cells involved in immune diseases. In a particular embodiment, the human antibodies are used to mediate killing of B lymphocytes in the treatment of lymphoma, *e.g.*, B cell non-Hodgkin's lymphoma.

Human antibodies of the invention include IgG1 (*e.g.*, IgG1,κ), IgG3 (*e.g.*, IgG3,κ) and IgG4 (*e.g.*, IgG4,κ) antibodies. However, other antibody isotypes are also encompassed by the invention, including IgG2, IgM, IgA1, IgA2, secretory IgA, IgD, and IgE. The antibodies can be whole antibodies or antigen-binding fragments thereof including, for example, Fab, $F(ab')_2$, Fv, single chain Fv fragments or bispecific antibodies. Furthermore, the antigen-binding fragments include binding-domain

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immunoglobulin fusion proteins comprising (i) a binding domain polypeptide (such as a heavy chain variable region or a light chain variable region) that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. Such binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939.

Particular human antibodies of the present invention include those referred to as 11B8, 2F2, and 7D8, encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof. In another embodiment, the human antibodies are characterized by having human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

In yet another embodiment, the human antibodies are characterized by having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively; SEQ ID NO:6 and SEQ ID NO:8, respectively; or SEQ ID NO:10 and SEQ ID NO:12, respectively.

Other particular human antibodies of the invention include those which comprise a CDR domain having a human heavy and light chain CDR1 region, a human heavy and light chain CDR2 region, and a human heavy and light chain CDR3 region, wherein

(a) the CDR1, CDR2, and CDR3 human heavy chain regions comprise an amino acid sequence selected from the group consisting of the amino acid sequences CDR1, CDR2, and CDR3 shown in Figures 53, 55, or 57 (SEQ ID NOs:13-15, 19-21, and 25-27), and conservative sequence modifications thereof, and

(b) the CDR1, CDR2, and CDR3 human light chain regions comprise an amino acid sequence selected from the group consisting of the amino acid sequences CDR1, CDR2, and CDR3 shown in Figures 53, 55, or 57 (SEQ ID NOs: 16-18, 22-24, and 28-30), and conservative sequence modifications thereof.

Also included within the present invention are antibodies which dissociate from CD20 with a dissociation equilibrium constant (K_D) of approximately 1-10 nM or less. Such antibodies also include those which do not cross-react with related cell-surface antigens and thus do not inhibit their function.

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In another embodiment, human anti-CD20 antibodies of the present invention can be characterized by one or more of the following properties:

a) specificity for human CD20;

b) a binding affinity to CD20 (K_D) of about 10 nM or less, preferably, about 5 nM or less and, more preferably, about 1-3 nM or less as determined by the binding experiment disclosed in Example 5 (Figure 9) herein;

c) a dissociation rate constant (k_d) from CD20 of about 10^{-4} sec^{-1} or less, preferably, about 10^{-5} sec^{-1} or less and, more preferably, about 10^{-6} sec^{-1} or less, as determined by the dissociation rate experiment disclosed in Example 5 (Figure 9) herein;

d) the ability to mediate a high level of CDC on either CD55/59 negative or CD55/59 positive cells;

e) the ability to translocate into lipid rafts upon binding to CD20;

f) the ability to inhibit the growth of cells which express CD20;

g) the ability to induce apoptosis of cells which express CD20;

h) the ability to induce homotypic adhesion of cells which express CD20;

i) the ability to induce ADCC of cells which express CD20 in the presence of effector cells;

j) the ability to prolong survival of a subject having tumor cells which express CD20;

k) the ability to deplete cells which express CD20; and/or

l) the ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

The human anti-CD20 antibodies of the present invention can be derivatized, linked to or co-expressed to other binding specificities. In a particular embodiment, the invention provides a bispecific or multispecific molecule comprising at least one first binding specificity for CD20 (*e.g.*, a human anti-CD20 antibody or mimetic thereof), and a second binding specificity for a human effector cell, such as a binding specificity for an Fc receptor (*e.g.*, a human Fcγ receptor, such as FcγRI, or a human Fcα receptor) or a T cell receptor, *e.g.*, CD3.

Accordingly, the present invention includes bispecific and multispecific molecules that bind to both human CD20 and to an Fc receptor or a T cell receptor, *e.g.*, CD3. Examples of Fc receptors are, *e.g.*, a human IgG receptor, *e.g.*, an Fc-gamma receptor (FcγR), such as FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). Other Fc receptors, such as human IgA receptors (*e.g.*, FcαRI), also can be targeted. The Fc receptor is preferably located on the surface of an effector cell, *e.g.*, a monocyte, macrophage or an activated mononuclear cell. In a preferred embodiment, the bispecific and multispecific molecules bind to an Fc receptor at a site which is distinct from the immunoglobulin Fc (*e.g.*, IgG or IgA) binding site of the receptor. Therefore, the

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binding of the bispecific and multispecific molecules is not blocked by physiological levels of immunoglobulins.

In yet another aspect, human anti-CD20 antibodies of the invention are derivatized, linked to or co-expressed with another functional molecule, *e.g.*, another peptide or protein (*e.g.*, a Fab' fragment). For example, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, to produce a bispecific or a multispecific antibody), a cytotoxin, cellular ligand or antigen (*e.g.*, to produce an immunoconjugate, such as an immunotoxin). An antibody of the present invention can be linked to other therapeutic moieties, *e.g.*, a radioisotope, a small molecule anti-cancer drug, an anti-inflammatory agent, or an immunosuppressive agent. Accordingly, the present invention encompasses a large variety of antibody conjugates, bispecific and multispecific molecules, and fusion proteins, all of which bind to CD20 expressing cells and which can be used to target other molecules to such cells.

In still another aspect, the invention provides compositions, *e.g.*, pharmaceutical and diagnostic compositions/kits, comprising a pharmaceutically acceptable carrier formulated along with one or a combination of human monoclonal antibodies of the invention. In a particular embodiment, the composition includes a combination of antibodies which bind to distinct epitopes or which possess distinct functional characteristics, such as inducing CDC and inducing apoptosis.

Human antibodies, immunoconjugates, bispecific and multispecific molecules and compositions of the present invention can be used in a variety of methods for inhibiting growth of cells expressing CD20 and/or killing cells expressing CD20 by contacting the cells with an effective amount of the antibody, immunconjugate, bispecific/multispecific molecule or composition, such that the growth of the cell is inhibited and/or the cell is killed. In one embodiment, the method includes killing of the cell expressing CD20 in the presence of effector cells, for example, by CDC, apoptosis, ADCC, phagocytosis, or by a combination of two or more of these mechanisms. The cells are preferably killed or inhibited without killing or inhibiting the activity of cells which do not express CD20 but which may, for example, express a structurally related cell-surface antigen (*i.e.*, without cross-reactivity to related but functionally distinct cell surface antigens). Cells expressing CD20 which can be inhibited or killed using the human antibodies of the invention include, for example, tumorigenic B cells.

Accordingly, human antibodies of the present invention can be used to treat and/or prevent a variety of diseases involving cells expressing CD20 by administering the antibodies to patients suffering from such diseases. Exemplary diseases that can be treated (*e.g.*, ameliorated) or prevented include, but are not limited

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to, tumorigenic diseases and immune diseases, *e.g.*, autoimmune diseases. Examples of tumorigenic diseases which can be treated and/or prevented include B cell lymphoma, *e.g.*, NHL, including precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), including low-grade, intermediate-grade and high-grade FL, cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL). Examples of immune disorders in which CD20 expressing B cells are involved which can be treated and/or prevented include psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases. Further examples are severe acute respiratory distress syndrome and choreoretinitis. Yet further examples are diseases and disorders caused by infection of B-cells with virus, such as Epstein-Barr virus (EBV).

In a particular embodiment of the invention, the subject being administered the antibody is additionally treated with a chemotherapeutic agent, radiation, or an agent that modulates, *e.g.*, enhances or inhibits, the expression or activity of an Fc receptor, *e.g.*, an Fcα receptor or an Fcγ receptor, such as a cytokine. Typical cytokines for administration during treatment include granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF). Typical therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide.

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In yet another aspect, the present invention provides a method for detecting *in vitro* or *in vivo* the presence of CD20 in a sample or individual, *e.g.*, for diagnosing a CD20-related disease, preferably at an early stage. This can also be useful for monitoring the disease and effect of treatment and for determining and adjusting the dose of the antibody to be administered. The *in vivo* method can be performed using imaging technique such as PET (positron emission tomography) or SPECT (single photon emission computed tomography). In one embodiment, this is achieved by contacting a sample to be tested, optionally along with a control sample, with a human monoclonal antibody of the invention under conditions that allow for formation of a complex between the antibody and CD20. Complex formation is then detected (*e.g.*, using an FACS analysis or Western blotting). When using a control sample along with the test sample, complex is detected in both samples and any statistically significant difference in the formation of complexes between the samples is indicative of the presence of CD20 in the test sample.

In yet another aspect, the invention provides a transgenic non-human animal, such as a transgenic mouse, which express human monoclonal antibodies that bind to CD20. In a particular embodiment, the transgenic non-human animal is a transgenic mouse having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an antibody of the invention. The transgenic non-human animal can be immunized with a purified or enriched preparation of CD20 antigen and/or cells expressing CD20. Preferably, the transgenic non-human animal, *e.g.*, the transgenic mouse, is capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgM) by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

Accordingly, in yet another aspect, the invention provides isolated B cells from a transgenic non-human animal as described above, *e.g.*, a transgenic mouse, which expresses human anti-CD20 antibodies. The isolated B cells can then be immortalized by fusion to an immortalized cell to provide a source (*e.g.*, a hybridoma) of human anti-CD20 antibodies. Such hybridomas (*i.e.*, which produce human anti-CD20 antibodies) are also included within the scope of the invention.

As exemplified herein, human antibodies of the invention can be obtained directly from hybridomas which express the antibody, or can be cloned and recombinantly expressed in a host cell (*e.g.*, a CHO cell, a NS/0 cell or a lymphocytic cell). Further examples of host cells are microorganisms, such as *E. coli*, and fungi, such as yeast. Alternatively, they can be produced recombinantly in a transgenic non-human animal or plant. Accordingly, in another aspect, the present invention provides methods for producing human monoclonal antibodies which bind to human CD20. In

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one embodiment, the method includes immunizing a transgenic non-human animal, *e.g.*, a transgenic mouse, as previously described (*e.g.*, having a genome comprising a human heavy chain transgene and a human light chain transgene encoding all or a portion of an anti-CD20 antibody), with a purified or enriched preparation of human CD20 antigen and/or cells expressing human CD20. B cells (*e.g.*, splenic B cells) of the animal are then obtained and fused with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies against CD20.

In yet another aspect, the invention provides nucleic acid molecules encoding human anti-CD20 antibodies (*e.g.*, variable regions thereof), as well as recombinant expression vectors which include the nucleic acids of the invention, and host cells transfected with such vectors. Methods of producing the antibodies by culturing these host cells are also encompassed by the invention. Particular nucleic acids provided by the invention comprise the nucleotide sequences shown in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, encoding the heavy and light chains, respectively, of human anti-CD20 antibodies 2F2, 7D8, and 11B8.

Other features and advantages of the instant invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the pCONγ1f/variable-heavy vector used for recombinant production of the human monoclonal antibodies 2F2 and 11B8.

Figure 2 shows the pCONκ/variable-light vector used for recombinant production of 2F2 and 11B8.

Figure 3 shows the double-gene cloning vector (pCONγ1f/κ2F2) used for recombinant production of 2F2 and 11B8.

Figure 4 is a graph comparing the binding of human monoclonal antibodies 2F2, 7D8, and 11B8 to Raji, Daudi, and CD20 transfected NS/0 cells and parental NS/0 cells using flow cytometry.

Figures 5A and 5B show the binding of 2F2 to PBMCs from three human donors using flow cytometry.

Figure 6 is a graph comparing the binding affinity of ^{125}I-labeled 2F2 and ^{125}I-labeled 11B8 to Ramos-EHRB cells.

Figures 7A and 7B show the binding of ^{125}I-labeled 2F2 and ^{125}I-labeled 11B8 compared to ^{125}I-labeled rituximab (chimeric anti-CD20 antibody, IDEC) and ^{125}I-labeled B1 (the term B1 corresponds to the unlabeled form of Bexxar™, which is a ^{131}I-labeled murine anti-human CD20 antibody, Coulter) to Ramos-EHRB cells (A) and Daudi cells (B).

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Figure 8 is a graph comparing the dissociation rates of ^{125}I-labeled 11B8T, ^{125}I-labeled 2F2, ^{125}I-labeled rituximab (RIT), and ^{125}I-labeled B1.

Figure 9 shows the dissociation rates of the F(ab')$_2$ fragments of 2F2, 11B8T, and rituximab in Ramos-EHRB cells.

Figures 10A and 10B show the CDC by 2F2T, 11B8T, 7D8, rituximab, and an isotype control antibody (HuMab-KLH) of Daudi cells (A) and SU-DHL-4 cells (B) at different time points (functional off-rate) using flow cytometry.

Figures 11A-E show the kinetics of CDC induced by 2F2 and rituximab in different cell lines using flow cytometry.

Figures 12A-D show CDC induced by 2F2 and rituximab in different cell lines as a function of the concentration of complement (normal human serum (NHS)) at two different antibody concentrations using flow cytometry.

Figures 13A-D show concentration-dependent induction of CDC by 2F2 and rituximab in different cell lines using flow cytometry.

Figures 14A and 14B show concentration-dependent induction of CDC by 2F2, 2F2T, 11B8T, B1, and rituximab in Daudi cells (A) and Raji cells (B).

Figures 15A and 15B are graphs comparing CDC of Daudi cells (cells expressing low levels of CD55/59) by human monoclonal antibodies 2F2, 7D8, and 11B8 and rituximab; (A) shows percent lysis of unwashed cells and (B) shows percent lysis of cells which were washed before the addition of serum.

Figures 16A and 16B are graphs comparing CDC of Raji cells (cells expressing high levels of CD55/59) by human monoclonal antibodies 2F2, 7D8, and 11B8 and rituximab; (A) shows percent lysis of cells not blocked with anti-CD55 and anti-CD59 antibodies, and (B) shows percent lysis of cells blocked with anti-CD55 and anti-CD59 antibodies.

Figures 17A-C show the role of CD55 and CD59 in CDC induced by 2F2 and rituximab in Raji cells. (A) shows the percentage of lysed cells upon addition of anti-CD55 antibody, (B) shows the percentage of lysed cells upon addition of anti-CD59 antibody, and (C) shows the percentage of lysed cells upon addition of both anti-CD55 antibody and anti-CD59 antibody.

Figures 18A-D show the binding of complement factor C1q by 2F2 and rituximab in different cell lines as determined by flow cytometry.

Figures 19A-D show the deposition of complement factor fragment C4c by 2F2 and rituximab in different cell lines as determined by flow cytometry.

Figure 20 shows lysis of ARH-77 cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 21 shows lysis of B-CLL cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

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Figure 22 shows lysis of HCL (hairy cell leukemia) cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 23 shows lysis of B-ALL cells by 2F2, and rituximab, in the presence of PMNs, MNCs, plasma or whole blood.

Figure 24 shows lysis of follicular lymphoma (FL) cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 25 shows lysis of mantle cell lymphoma cells by 2F2, rituximab, and 11B8T in the presence of PMNs, MNCs, plasma or whole blood.

Figure 26 shows concentration dependent lysis of ARH-77 cells by 2F2 and rituximab in the presence of whole blood.

Figure 27 shows MNC-mediated lysis of ARH-77 cells by 2F2T, 11B8T, and rituximab.

Figure 28 shows MNC-mediated lysis of Raji cells by 2F2T, 11B8T, and rituximab.

Figures 29A, B, and C are graphs showing clustering of CD20 in the lipid rafts upon incubation with 2F2, 7D8, or 11B8 using FRET analysis and Triton-X insolubility assay.

Figure 30 shows clustering of CD20 in the lipid rafts upon incubation with 2F2, rituximab, or 11B8T using FRET analysis.

Figure 31 shows the proportion of CD20 remaining in the insoluble raft fraction after treatment with Triton X-100 (TX) and incubation with 2F2, rituximab, or 11B8T.

Figure 32 shows the distribution of CD20 between the raft and non-raft membrane fractions upon stimulating Daudi cells with 2F2, rituximab, or 11B8T.

Figures 33A-G show apoptosis of Daudi cells by 2F2, 7D8, and 11B8 using flow cytometry.

Figure 34 shows induction of apoptosis of Raji cells by 2F2, 11B8T, rituximab, or B1 using flow cytometry.

Figure 35A shows induction of apoptosis of Daudi cells by 2F2T, 11B8T, rituximab, or B1 using flow cytometry.

Figure 35B shows early stage and late stage apoptosis of Daudi cells by human monoclonal antibodies 2F2T, 11B8T, rituximab, and B1 using flow cytometry.

Figures 36A-E show homotypic adhesion of Ramos-EHRB cells by 2F2, 7D8, and 11B8 using light microscopy.

Figure 37 show homotypic adhesion of Daudi cells by 2F2, rituximab, and B1 using light microscopy.

Figure 38 is a graph showing the percent survival of SCID mice injected with Daudi cells and treated with 2F2 or 7D8.

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Figure 39 shows the percent survival of SCID mice injected with Tanoue cells and treated with 2F2, rituximab, or B1.

Figure 40 shows the percent survival of SCID mice injected with Daudi cells and treated with different concentrations of 2F2 or rituximab.

Figure 41 shows the percent survival of SCID mice injected with Daudi cells and treated with 11B8T or B1.

Figure 42 shows bioluminescence imaging of tumor cells in SCID mice on day 39 (31 days after treatment with 10 μg of B1, rituximab, 11B8T, 2F2T, or huIgG1). The bioluminescence is represented in red color (the dark areas in the mice) (light intensity > 50 photons per 5 min) as overlay on the black and white body image of the mice.

Figure 43 shows the tumor mass in each mouse quantified on day 25, 32, 39, and 46 following administration, on day 8, of 10 μg of B1, rituximab, 11B8T, 2F2T, or huIgG1 by integrating the light signals over the body surface.

Figures 44A-C show flow cytometric analysis of $CD20^+$ cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 45A-C show flow cytometric analysis of $CD21^+$ cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 46A-C show flow cytometric analysis of $CD20^+$ cells in lymph node of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 47A-C show flow cytometric analysis of $CD20^{low}CD23^+CD40^{high}$ expressing cells in peripheral blood of cynomolgus monkeys following intravenous administration of 2F2 or rituximab at different dosages, 4 x 1.25 mk/kg (A), 4 x 6.25 mg/kg (B), or 4 x 12.50 mg/kg (C).

Figures 48A-E show binding of rituximab (A), 2F2 (B), 11B8 (C), B1 (D), or an isotype control antibody (E) to CHO cells expressing wild type (WT) CD20, mutant CD20 (AxP), or both WT CD20 and mutant CD20 (AxP) as determined by flow cytometry.

Figures 49A-F show percentage binding of 2F2, 11B8T, B1 or rituximab to mutant P172S vs. WT CD20 (A), percentage binding of 2F2T, 11B8T, B1, CAT (CAT 13.6E12, a mouse monoclonal IgG2A anti-CD20 antibody, Diatec.Com), a control isotype antibody (KLH) or rituximab to mutant CD20 (AxP) vs.

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WT CD20 (B), percentage binding of 2F2, 11B8T, B1 or rituximab to mutant N166D vs. WT CD20 (C), percentage binding of 2F2T, CAT or rituximab to mutant N166D vs. WT CD20 (D) , percentage binding of 2F2T, 2F2, 11B8T, B1 or rituximab to mutant N163D vs. WT CD20 (E), and percentage binding of 2F2T, CAT or rituximab to mutant N163D vs. WT CD20 (F).

Figure 50 shows binding of 2F2T, 7D8, and isotype control antibody, as determined by ELISA, to three anti-idiotypic antibodies, anti-2F2 sab 1.1, anti-2F2 sab 1.2, and anti-2F2 sab 1.3, raised against 2F2.

Figure 51 shows binding of 11B8T, as determined by ELISA, to anti-idiotypic antibodies, anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6, raised against 11B8T, but no binding to the anti-idiotypic anti-2F2 antibodies.

Figures 52A-C show dose-dependent binding of 2F2T, as determined by ELISA, to three anti-idiotypic antibodies, anti-2F2 sab 1.1 (A), anti-2F2 sab 1.2 (B), and anti-2F2 sab 1.3 (C), raised against 2F2.

Figure 53 shows the amino acid sequence (SEQ ID NO:2) of the heavy chain V region and the amino acid sequence (SEQ ID NO:4) of the light (kappa) chain V region of human monoclonal antibody 2F2 with CDR regions designated.

Figure 54 shows the nucleotide sequence (SEQ ID NO:1) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:3) of the light (kappa) chain V region of human monoclonal antibody 2F2.

Figure 55 shows the amino acid sequence (SEQ ID NO:6) of the heavy chain V region and the amino acid sequence (SEQ ID NO:8) of the light (kappa) chain V region of human monoclonal antibody 7D8 with CDR regions designated.

Figure 56 shows the nucleotide sequence (SEQ ID NO:5) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:7) of the light (kappa) chain V region of human monoclonal antibody 7D8.

Figure 57 shows the amino acid sequence (SEQ ID NO:10) of the heavy chain V region and the amino acid sequence (SEQ ID NO:12) of the light (kappa) chain V region of human monoclonal antibody 11B8 with CDR regions designated.

Figure 58 shows the nucleotide sequence (SEQ ID NO:9) of the heavy chain V region and the nucleotide sequence (SEQ ID NO:11) of the light (kappa) chain V region of human monoclonal antibody 11B8.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved antibody-based therapies for treating and diagnosing a variety of disorders involving cells expressing CD20. Therapies of the invention employ isolated human monoclonal antibodies which

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specifically bind to an epitope present on CD20. Isolated human monoclonal antibodies encompassed by the present invention include IgA, IgG1-4, IgE, IgM, and IgD antibodies.

In one embodiment the antibody is an IgG1 antibody, more particularly an IgG1,κ or IgG1,λ isotype. In another embodiment the antibody is an IgG3 antibody, more particularly an IgG3,κ or IgG3,λ isotype. In yet another embodiment the antibody is an IgG4 antibody, more particularly an IgG4,κ or IgG4,λ isotype. In still another embodiment the antibody is an IgA1 or IgA2 antibody.

In still another embodiment the antibody is an IgM antibody.

In one embodiment, the human antibodies are produced in a non-human transgenic animal, *e.g.*, a transgenic mouse, capable of producing multiple isotypes of human monoclonal antibodies to CD20 by undergoing V-D-J recombination and isotype switching. Accordingly, aspects of the invention include not only antibodies, antibody fragments, and pharmaceutical compositions thereof, but also non-human transgenic animals, B cells, host cell transfectomas, and hybridomas which produce monoclonal antibodies. Such transgenic animal can also be a transgenic rabbit for producing polyclonal antibodies such as disclosed in US 2003/0017534. Accordingly, the invention also encompasses human polyclonal antibodies which specifically bind to CD20. In one embodiment the invention relates to polyclonal antibodies which bind to an epitope on CD20 (i) which does not comprise or require the amino acid residue proline at position 172; (ii) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172; (iii) which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; (iv) which does not comprise or require the amino acid residue proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; or (v) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166.

In another embodiment the invention relates to human polyclonal antibodies which have one or more of the following characteristics: (i) bind to mutant P172S CD20 (proline at position 172 mutated to serine) with at least the same affinity as to human CD20; (ii) bind to mutant AxP (alanine at position 170 mutated to serine, and proline at position 172 mutated to serine) with at least the same affinity as to human CD20; (iii) show a reduced binding of 50% or more to mutant N166D (asparagine at position 166 mutated to aspartic acid) to human CD20 at an antibody concentration of 10 μg/ml; and/or (iv) show a reduced binding of 50% or more to mutant N163D (asparagine at position 163 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml.

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In yet another embodiment the invention the invention relates to human polyclonal antibodies which bind to an epitope in the small first extracellular loop of human CD20. In still another embodiment the invention also encompasses human polyclonal antibodies which bind to a discontinuous epitope on CD20. In a further embodiment the invention relates to human polyclonal antibodies which bind a discontinuous epitope on CD20, which has part of the first small extracellular loop and part of the second extracellular loop. In still a further embodiment the invention relates to human polyclonal antibodies which bind to a discontinuous epitope on CD20, which has residues AGIYAP of the small first extracellular loop and residues MESLNFIRAHTPYI of the second extracellular loop.

Methods of using the antibodies of the invention to detect a cell expressing CD20 are encompassed by the invention. Methods of using the antibodies of the invention to block or inhibit CD20 induced activities, *e.g.*, proliferative and/or differentiation activities, are also provided and are useful in the treatment of disorders associated with CD20, such as tumorigenic diseases (*e.g.*, B cell lymphoma) and autoimmune diseases (*e.g.*, RA, Chrohn's disease and Wegener's granulomatosis).

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The terms "CD20" and "CD20 antigen" are used interchangeably herein, and include any variants, isoforms and species homologs of human CD20 which are naturally expressed by cells or are expressed on cells transfected with the CD20 gene. Binding of an antibody of the invention to the CD20 antigen mediate the killing of cells expressing CD20 (*e.g.*, a tumor cell) by inactivating CD20. The killing of the cells expressing CD20 may occur by one or more of the following mechanisms:

complement dependent cytotoxity (CDC) of cells expressing CD20;
apoptosis of cells expressing CD20;
effector cell phagocytosis of cells expressing CD20; or
effector cell antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20.

Synonyms of CD20, as recognized in the art, include B-lymphocyte antigen CD20, B-lymphocyte surface antigen B1, Leu-16, Bp35, BM5, and LF5.

As used herein, the term "inhibits growth" (*e.g.*, referring to cells) is intended to include any measurable decrease in the cell growth when contacted with an anti-CD20 antibody as compared to the growth of the same cells not in contact with an anti-CD20 antibody, *e.g.*, the inhibition of growth of a cell culture by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 99%, or 100%. Such a decrease in cell

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growth can occur by a variety of mechanisms, *e.g.*, effector cell phagocytosis, ADCC, CDC, and/or apoptosis.

The term "raft" refers to the sphingolipid- and cholesterol-rich membrane microdomains located in the outer leaflet area of the plasma membrane of a cell. The ability of certain proteins to associate within such domains can effect the protein's function. For example, the translocation of CD20 molecules into lipid rafts, after being bound by human antibodies of the present invention, creates a high density of CD20 antigen-antibody complexes in the plasma membranes. Such a high density of CD20 antigen-antibody complexes can enable efficient activation of the complement system during CDC.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, CD20). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; (vi) an isolated complementarity determining region (CDR), and (vii) a combination of two or more

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isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883).* Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. A further example is binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. The binding domain polypeptide can be a heavy chain variable region or a light chain variable region. The binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term "discontinuous epitope," as used herein, means a conformational epitope on a protein antigen which is formed from at least two separate regions in the primary sequence of the protein.

The term "bispecific molecule" is intended to include any agent, *e.g.*, a protein, peptide, or protein or peptide complex, which has two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen and (b) an Fc receptor on the surface of an effector cell. The term "multispecific molecule" or "heterospecific molecule" is intended to include any agent, *e.g.*, a protein, peptide, or protein or peptide complex, which has more than two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen, (b) an Fc receptor on the surface of an effector cell, and (c) at least one other component. Accordingly, the invention includes, but is not limited to, bispecific, trispecific, tetraspecific, and other multispecific molecules which are directed to cell

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surface antigens, such as CD20, and to other targets, such as Fc receptors on effector cells.

The term "bispecific antibodies" also includes diabodies. Diabodies are bivalent, bispecific antibodies in which the V_H and V_L domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123).

The term "human antibody derivatives" refers to any modified form of the antibody, *e.g.*, a conjugate of the antibody and another agent or antibody.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, *e.g.*, by immunizing a transgenic mouse carrying human immunoglobulin genes or by screening a human immunoglobulin gene library, and wherein the selected human antibody is at least 90%, more preferably at least 95%, even more preferably at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid differences, more preferably, no more than 5, or even more preferably, no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

As used herein, the term "heteroantibodies" refers to two or more antibodies, derivatives therefrom, or antigen binding regions linked together, at least two of which have different specificities. These different specificities include a binding specificity for an Fc receptor on an effector cell, and a binding specificity for an antigen or epitope on a target cell, *e.g.*, a tumor cell.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

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The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further in Section I, below), (b) antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term “transfectoma”, as used herein, includes recombinant eukaryotic host cell expressing the antibody, such as CHO cells, NS/0 cells, HEK293 cells, plant cells, or fungi, including yeast cells .

As used herein, a "heterologous antibody" is defined in relation to the transgenic non-human organism producing such an antibody. This term refers to an antibody having an amino acid sequence or an encoding nucleic acid sequence corresponding to that found in an organism not consisting of the transgenic non-human animal, and generally from a species other than that of the transgenic non-human animal.

As used herein, a "heterohybrid antibody" refers to an antibody having a light and heavy chains of different organismal origins. For example, an antibody having a human heavy chain associated with a murine light chain is a heterohybrid antibody.

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Examples of heterohybrid antibodies include chimeric and humanized antibodies, discussed supra.

An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds to CD20 is substantially free of antibodies that specifically bind antigens other than CD20). An isolated antibody that specifically binds to an epitope, isoform or variant of human CD20 may, however, have cross-reactivity to other related antigens, *e.g.*, from other species (*e.g.*, CD20 species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different specificities are combined in a well defined composition.

As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity corresponding to a K_D of about 1 x 10^{-7} M or less, and binds to the predetermined antigen with an affinity corresponding to a K_D that is at least two orders of magnitude lower than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and " an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

As used herein, the term "k_d" (sec^{-1}), as used herein, is intended to refer to the dissociation rate constant of a particular antibody-antigen interaction. Said value is also referred to as the k_{off} value.

The term "k_a" (M^{-1} x sec^{-1}), as used herein, is intended to refer to the association rate constant of a particular antibody-antigen interaction.

The term "K_D" (M), as used herein, is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction.

The term "K_A" (M^{-1}), as used herein, is intended to refer to the association equilibrium constant of a particular antibody-antigen interaction and is obtained by dividing the k_a by the k_d.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by heavy chain constant region genes.

As used herein, "isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

As used herein, "nonswitched isotype" refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the CH gene encoding the nonswitched isotype is typically the first CH gene immediately

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downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination events which involve at least one switch sequence region in the transgene. Non-classical isotype switching may occur by, for example, homologous recombination between human σ_μ and human $\sum_\mu$ (δ-associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

As used herein, the term "switch sequence" refers to those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, will be 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region will be between the construct region to be deleted and the replacement constant region (*e.g.*, γ, ε, etc.). As there is no specific site where recombination always occurs, the final gene sequence will typically not be predictable from the construct.

As used herein, "glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin (antibody) protein. A glycosylation pattern of a heterologous antibody can be characterized as being substantially similar to glycosylation patterns which occur naturally on antibodies produced by the species of the non-human transgenic animal, when one of ordinary skill in the art would recognize the glycosylation pattern of the heterologous antibody as being more similar to said pattern of glycosylation in the species of the non-human transgenic animal than to the species from which the CH genes of the transgene were derived.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "rearranged" as used herein refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete V_H or V_L domain, respectively. A rearranged immunoglobulin (antibody) gene locus can be identified by comparison to germline DNA; a rearranged locus will have at least one recombined heptamer/nonamer homology element.

The term "unrearranged" or "germline configuration" as used herein in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

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The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding whole antibodies or antibody portions (*e.g.*, V_H, V_L, CDR3) that bind to CD20, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the intact antibody or antibody portion are free of other nucleotide sequences encoding whole antibodies or antibody portions that bind antigens other than CD20, which other sequences may naturally flank the nucleic acid in human genomic DNA. In one embodiment, the human anti-CD20 antibody includes the nucleotide or amino acid sequence of 2F2, 7D8, or 11B8, as well as heavy chain (V_H) and light chain (V_L) variable regions having the sequences shown in SEQ ID NOs: 1, 5, or 9, and SEQ ID NOs: 3, 7, or 11, respectively.

As disclosed and claimed herein, the sequences set forth in SEQ ID NOs: 1-30 include "conservative sequence modifications," *i.e.*, nucleotide and amino acid sequence modifications which do not significantly affect or alter the binding characteristics of the antibody encoded by the nucleotide sequence or containing the amino acid sequence. Such conservative sequence modifications include nucleotide and amino acid substitutions, additions and deletions. Modifications can be introduced into SEQ ID NOs:1-30 by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-CD20 antibody is preferably replaced with another amino acid residue from the same side chain family.

The present invention also encompasses "derivatives" of the amino acid sequences as set forth in SEQ ID NOs: 1-30 and conservative sequence modifications thereof, wherein one or more of the amino acid residues have been derivatised, *e.g.*, by acylation or glycosylation, without significantly affecting or altering the binding characteristics of the antibody containing the amino acid sequences.

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Furthermore, the present invention comprises antibodies in which alterations have been made in the Fc region in order to change the functional or pharmacokinetic properties of the antibodies. Such alterations may result in a decrease or increase of C1q binding and CDC or of FcγR binding and ADCC. Substitutions can, for example, be made in one or more of the amino acid residues at positions 234, 235, 236, 237, 297, 318, 320 and 322 of the heavy chain constant region, thereby causing an alteration in an effector function while retaining the ability to bind to the antigen as compared with the unmodified antibody, cf. US 5,624,821 and US 5,648,260.

The *in vivo* half-life of the antibodies can also be improved by modifying the salvage receptor epitope of the Ig constant domain or an Ig-like constant domain such that the molecule does not comprise an intact CH2 domain or an intact Ig Fc region, cf. US 6,121,022 and US 6,194,551. The *in vivo* half-life can furthermore be increased by making mutations in the Fc region, *e.g.*, by substituting threonine for leucine at position 252, by substituting threonine for serine at position 254, or by substituting threonine for phenylalanine at position 256, cf. US 6,277,375.

Furthermore, the glycosylation pattern of the antibodies can be modified in order to change the effector function of the antibodies. For example, the antibodies can be expressed in a transfectoma which does not add the fucose unit normally attached to Asn at position 297 of the Fc region in order to enhance the affinity of the Fc region for FcγRIII which, in turn, will result in an increased ADCC of the antibodies in the presence of NK cells, cf. Shield *et al.* (2002) *JBC*, 277:26733. Furthermore, modification of galactosylation can be made in order to modify CDC.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a anti-CD20 antibody coding sequence, such as by saturation mutagenesis, and the resulting modified anti-CD20 antibodies can be screened for binding activity.

Accordingly, antibodies encoded by the (heavy and light chain variable region) nucleotide sequences disclosed herein and/or containing the (heavy and light chain variable region) amino acid sequences disclosed herein (*i.e.*, SEQ ID NOs: 1-30) include substantially similar antibodies encoded by or containing similar sequences which have been conservatively modified. Further discussion as to how such substantially similar antibodies can be generated based on the partial (*i.e.*, heavy and light chain variable regions) sequences disclosed herein as SEQ ID Nos:1-30 is provided below.

For nucleic acids, the term "substantial homology" indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about

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98% to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

For nucleotide and amino acid sequences, the term "homology" indicates the degree of identity between two nucleic acid or amino acid sequences when optimally aligned and compared with appropriate insertions or deletions. Alternatively, substantial homology exists when the DNA segments will hybridize under selective hybridization conditions, to the complement of the strand.

The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*,

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(1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See*, F. Ausubel, *et al.*, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures thereof, may be mutated in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription of regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors").

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In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. Recombinant host cells include, for example, transfectomas, such as CHO cells, NS/0 cells, and lymphocytic cells.

As used herein, the term "subject" includes any human or non-human animal. The term "non-human animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

The terms "transgenic, non-human animal" refers to a non-human animal having a genome comprising one or more human heavy and/or light chain transgenes or transchromosomes (either integrated or non-integrated into the animal's natural genomic DNA) and which is capable of expressing fully human antibodies. For example, a transgenic mouse can have a human light chain transgene and either a human heavy chain transgene or human heavy chain transchromosome, such that the mouse produces human anti-CD20 antibodies when immunized with CD20 antigen and/or cells expressing CD20. The human heavy chain transgene can be integrated into the chromosomal DNA of the mouse, as is the case for transgenic, *e.g.*, HuMAb mice, such as HCo7 or HCo12 mice, or the human heavy chain transgene can be maintained extrachromosomally, as is the case for transchromosomal (*e.g.*, KM) mice as described in WO 02/43478. Such transgenic and transchromosomal mice are capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching.

Various aspects of the invention are described in further detail in the following subsections.

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I. Production of Human Antibodies to CD20

Human monoclonal antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed, *e.g.*, viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of human antibody genes.

The preferred animal system for preparing hybridomas that secrete human monoclonal antibodies is the murine system. Hybridoma production in the mouse is a very well established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

In a preferred embodiment, human monoclonal antibodies directed against CD20 can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as HuMAb mice and KM mice, respectively, and are collectively referred to herein as "transgenic mice."

The HuMAb mouse contains a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. *et al.* (1994) *Nature* 368 (6474): 856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ and in response to immunization, the introduced human heavy and light chain transgenes, undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal antibodies (Lonberg, N. *et al.* (1994), supra; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764: 536-546). The preparation of HuMAb mice is described in detail in Taylor, L. *et al.* (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. et al (1993) *International Immunology* 5: 647-656; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912-2920; Lonberg *et al.*, (1994) *Nature* 368(6474): 856-859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylor, L. *et al.* (1994) *International Immunology* 6: 579-591; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13:65-93; Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546; Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14:845-851. See further, US Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay, as well as US 5,545,807 to Surani *et al.*; WO

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98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918 and WO 01/09187.

The KM mouse contains a human heavy chain transchromosome and a human kappa light chain transgene. The endogenous mouse heavy and light chain genes also have been disrupted in the KM mice such that immunization of the mice leads to production of human immunoglobulins rather than mouse immunoglobulins. Construction of KM mice and their use to raise human immunoglobulins is described in detail in WO 02/43478.

Immunizations

To generate fully human monoclonal antibodies to CD20, transgenic or transchromosomal mice containing human immunoglobulin genes (*e.g.*, HCo12, HCo7 or KM mice) can be immunized with an enriched preparation of CD20 antigen and/or cells expressing CD20, as described, for example, by Lonberg *et al.* (1994), supra; Fishwild *et al.* (1996), *supra*, and WO 98/24884. Alternatively, mice can be immunized with DNA encoding human CD20. Preferably, the mice will be 6-16 weeks of age upon the first infusion. For example, an enriched preparation (5-50 μg) of the CD20 antigen can be used to immunize the HuMAb mice intraperitoneally. In the event that immunizations using a purified or enriched preparation of the CD20 antigen do not result in antibodies, mice can also be immunized with cells expressing CD20, *e.g.*, a cell line, to promote immune responses.

Cumulative experience with various antigens has shown that the HuMAb transgenic mice respond best when initially immunized intraperitoneally (IP) or subcutaneously (SC) with CD20 expressing cells in complete Freund's adjuvant, followed by every other week IP immunizations (up to a total of 10) with CD20 expressing cells in PBS. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by FACS analysis (as described below), and mice with sufficient titers of anti-CD20 human immunoglobulin can be used for fusions. Mice can be boosted intravenously with CD20 expressing cells 3 days before sacrifice and removal of the spleen.

Generation of Hybridomas Producing Human Monoclonal Antibodies to CD20

To generate hybridomas producing human monoclonal antibodies to human CD20, splenocytes and lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can then be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from

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immunized mice can be fused to SP2/0-Ag8.653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50% PEG (w/v). Cells can be plated at approximately 1 x 10^5 per well in flat bottom microtiter plate, followed by a two week incubation in selective medium containing besides usual reagents 10% fetal Clone Serum, 5-10% origen hybridoma cloning factor (IGEN) and 1X HAT (Sigma). After approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human kappa-light chain containing antibodies and by FACS analysis using CD20 expressing cells for CD20 specificity. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again, and if still positive for human IgG, anti-CD20 monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured *in vitro* to generate antibody in tissue culture medium for characterization.

Generation of Transfectomas Producing Human Monoclonal Antibodies to CD20

Human antibodies of the invention also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art (Morrison, S. (1985) Science 229:1202).

For example, in one embodiment, the gene(s) of interest, *e.g.*, human antibody genes, can be ligated into an expression vector such as a eukaryotic expression plasmid such as used by the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338 841 or other expression systems well known in the art. The purified plasmid with the cloned antibody genes can be introduced in eukaryotic host cells such as CHO cells, NS/0 cells or HEK293 cells or alternatively other eukaryotic cells like a plant derived cells, fungi or yeast cells. The method used to introduce these genes could be methods described in the art such as electroporation, lipofectine, lipofectamine or other. After introducing these antibody genes in the host cells, cells expressing the antibody can be identified and selected. These cells represent the transfectomas which can then be amplified for their expression level and upscaled to produce antibodies. Recombinant antibodies can be isolated and purified from these culture supernatants and/or cells.

Further Recombinant Means for Producing Human Monoclonal Antibodies to CD20

Alternatively, the cloned antibody genes can be expressed in other expression systems, including prokaryotic cells, such as microorganisms, such as *E. coli*, for the production of single chain Fv antibodies, algi, as well as insect cells. Furthermore, the antibodies can be produced in transgenic non-human animals, such as

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in milk from sheep and rabbits or in eggs from hens, or in transgenic plants. See *e.g.* Verma, R., *et al.* (1998). Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression systems. *J.Immunol.Meth.* 216:165-181; Pollock, *et al.* (1999). Transgenic milk as a method for the production of recombinant antibodies. *J.Immunol.Meth.* 231:147-157; and Fischer, R., *et al.* (1999). Molecular farming of recombinant antibodies in plants. *Biol.Chem.* 380:825-839.

Use of Partial Antibody Sequences to Express Intact Antibodies

Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.* (1998) *Nature* 332:323-327; Jones, P. *et al.* (1986) *Nature* 321:522-525; and Queen, C. *et al.* (1989) *Proc. Natl. Acad. Sci.*. U.S.A. 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequences of a high affinity secondary repertoire antibody at individual evenly across the variable region. For example, somatic mutations are relatively infrequent in the amino terminal portion of framework region 1 and in the carboxy-terminal portion of framework region 4. Furthermore, many somatic mutations do not significantly alter the binding properties of the antibody. For this reason, it is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody (see WO 99/45962). Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in missing portions of the variable regions. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire variable

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region can be synthesized as a set of short, overlapping, oligonucleotides and combined by PCR amplification to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion or particular restriction sites, or optimization of particular codons.

The nucleotide sequences of heavy and light chain transcripts from hybridomas are used to design an overlapping set of synthetic oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and kappa chain sequences can differ from the natural sequences in three ways: strings of repeated nucleotide bases are interrupted to facilitate oligonucleotide synthesis and PCR amplification; optimal translation initiation sites are incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266:19867-19870); and HindIII sites are engineered upstream of the translation initiation sites.

For both the heavy and light chain variable regions, the optimized coding and corresponding non-coding, strand sequences are broken down into 30 - 50 nucleotides approximately at the midpoint of the corresponding non-coding oligonucleotide. Thus, for each chain, the oligonucleotides can be assembled into overlapping double stranded sets that span segments of 150 - 400 nucleotides. The pools are then used as templates to produce PCR amplification products of 150 - 400 nucleotides. Typically, a single variable region oligonucleotide set will be broken down into two pools which are separately amplified to generate two overlapping PCR products. These overlapping products are then combined by PCR amplification to form the complete variable region. It may also be desirable to include an overlapping fragment of the heavy or light chain constant region (including the BbsI site of the kappa light chain, or the AgeI site if the gamma heavy chain) in the PCR amplification to generate fragments that can easily be cloned into the expression vector constructs.

The reconstructed heavy and light chain variable regions are then combined with cloned promoter, leader sequence, translation initiation, constant region, 3' untranslated, polyadenylation, and transcription termination, sequences to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a host cell expressing both chains.

Plasmids for use in construction of expression vectors for human IgGκ are described below. The plasmids were constructed so that PCR amplified V heavy and V kappa light chain cDNA sequences could be used to reconstruct complete heavy and light chain minigenes. These plasmids can be used to express completely human, or chimeric IgG1,κ or IgG4,κ antibodies. Similar plasmids can be constructed for

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expression of other heavy chain isotypes, or for expression of antibodies comprising lambda light chains.

Thus, in another aspect of the invention, the structural features of the human anti-CD20 antibodies of the invention, *e.g.*, 11B8, 2F2, or 7D8, are used to create structurally related human anti-CD20 antibodies that retain at least one functional property of the antibodies of the invention, such as binding to CD20. More specifically, one or more CDR regions of 2F2, 7D8, or 11B8 can be combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, human anti-CD20 antibodies of the invention.

Accordingly, in another embodiment, the invention provides a method for preparing an anti-CD20 antibody comprising:

preparing an antibody comprising (1) human heavy chain framework regions and human heavy chain CDRs, wherein at least one of the human heavy chain CDRs comprises an amino acid sequence selected from the amino acid sequences of CDRs shown in Figures 53, 55, or 57 (or corresponding amino acid residues in SEQ ID NOs:13-15, 19-21, or 25-27); and (2) human light chain framework regions and human light chain CDRs, wherein at least one of the human light chain CDRs comprises an amino acid sequence selected from the amino acid sequences of CDRs shown in Figures 53, 55, or 57 (or corresponding amino acid residues in SEQ ID NOs: 16-18, 22-24, or 28-30); wherein the antibody retains the ability to bind to CD20.

The ability of the antibody to bind CD20 can be determined using standard binding assays, such as those set forth in the Examples (*e.g.*, a FACS analysis).

Since it is well known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen, the recombinant antibodies of the invention prepared as set forth above preferably comprise the heavy and light chain CDR3s of 2F2, 7D8, or 11B8. The antibodies further can comprise the CDR2s of 2F2, 7D8, or 11B8. The antibodies further can comprise the CDR1s of 2F2, 7D8, or 11B8. Accordingly, the invention further provides anti-CD20 antibodies comprising: (1) human heavy chain framework regions, a human heavy chain CDR1 region, a human heavy chain CDR2 region, and a human heavy chain CDR3 region, wherein the human heavy chain CDR3 region is the CDR3 of 2F2, 7D8, or 11B8 as shown in Figures 53, 55, or 57 (or corresponding amino acid residues as shown in SEQ ID NOs: 15, 21, or 27); and (2) human light chain framework regions, a human light chain CDR1 region, a human light chain CDR2 region, and a human light chain CDR3 region, wherein the human light chain CDR3 region is the CDR3 of 2F2, 7D8, or 11B8 as shown in Figures 53, 55, or 57 (or corresponding amino acid residues as shown in SEQ ID NOs:18, 24, or 30), wherein the antibody binds CD20. The antibody may further comprise the heavy chain CDR2 and/or

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the light chain CDR2 of 2F2, 7D8, or 11B8. The antibody may further comprise the heavy chain CDR1 and/or the light chain CDR1 of 2F2, 7D8, or 11B8.

Preferably, the CDR1, 2, and/or 3 of the engineered antibodies described above comprise the exact amino acid sequence(s) as those of 2F2, 7D8, or 11B8 disclosed herein. However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences of 2F2, 7D8, or 11B8 may be possible while still retaining the ability of the antibody to bind CD20 effectively (*e.g.*, conservative substitutions). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 90%, 95%, 98% or 99.5% identical to one or more CDRs of 2F2, 7D8, or 11B8.

In addition to simply binding CD20, engineered antibodies such as those described above may be selected for their retention of other functional properties of antibodies of the invention, such as:

(1) low dissociation rate from CD20;

(2) high affinity binding to CD20;

(3) binding to a unique epitope on CD20, and/or binding in a specific orientation to CD20, and/or binding to a specific form of CD20;

(4) mediation of a high level of CDC on either CD55/59 negative or CD55/59 positive cells;

(5) translocation into lipid rafts upon binding to CD20;

(6) inhibition of the growth of cells which express CD20;

(7) inducement of apoptosis of cells which express CD20;

(8) inducement of homotypic adhesion of cells which express CD20;

(9) prolonged survival of a subject having tumor cells which express CD20;

(10) mediation of ADCC of CD20 targets when mixed with appropriate effector cells;

(11) ability to deplete cells which express CD20; and/or

(12) ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

Characterization of Binding of Human Monoclonal Antibodies to CD20

To purify human anti-CD20 antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (for IgG1 isotype antibodies) (Pharmacia, Piscataway, NJ) or anti-human IgG coated sepharose or protein G-sepharose in case of IgG3 isotype antibodies. Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to

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ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD_{280} using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80 °C.

To determine if the selected human anti-CD20 monoclonal antibodies bind to unique epitopes, site-directed or multi-site directed mutagenesis can be used.

To determine the isotype of purified antibodies, isotype ELISAs can be performed. Wells of microtiter plates can be coated with 10 μg/ml of anti-human Ig overnight at 4 °C. After blocking with 5% BSA, the plates are reacted with 10 μg/ml of monoclonal antibodies or purified isotype controls, at ambient temperature for two hours. The wells can then be reacted with either human IgGl, IgG2, IgG3 or IgG4 or human IgM-specific alkaline phosphatase-conjugated probes. After washing, the plates are developed with pNPP substrate (1 mg/ml) and analyzed at OD of 405-650.

In order to demonstrate presence of anti-CD20 antibodies in sera of immunized mice or binding of monoclonal antibodies to live cells expressing the CD20, flow cytometry can be used. Briefly, cell lines expressing CD20 (grown under standard growth conditions) are mixed with various concentrations of monoclonal antibodies in PBS containing 0.1% BSA and 0.02% sodium-azide, and incubated at 4 °C for 30 min. After washing, the cells are reacted with fluorescein-labeled anti-human IgG antibody under the same conditions as the primary antibody staining. The samples can be analyzed by flow cytometry with a FACS instrument using light and side scatter properties to gate on single, living cells. An alternative assay using fluorescence microscopy may be used (in addition to or instead of) the flow cytometry assay. Cells can be stained exactly as described above and examined by fluorescence microscopy. This method allows visualization of individual cells, but may have diminished sensitivity depending on the density of the antigen.

Anti-CD20 human IgGs can be further tested for reactivity with CD20 antigen by Western blotting. Briefly, cell extracts from cells expressing CD20 can be prepared and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens will be transferred to nitrocellulose membranes, blocked with 20% mouse serum, and probed with the monoclonal antibodies to be tested. Human IgG binding can be detected using anti-human IgG alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, MO).

Phagocytic and Cell Killing Activities of Human Monoclonal Antibodies to CD20

In addition to binding specifically to CD20, human monoclonal anti-CD20 antibodies can be tested for their ability to mediate phagocytosis and killing of cells expressing CD20. The testing of monoclonal antibody activity *in vitro* will provide

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an initial screening prior to testing *in vivo* models. Briefly, polymorphonuclear cells (PMNs), NK cells, monocytes or other effector cells, from healthy donors can be purified by Ficoll Hypaque density centrifugation, followed by lysis of contaminating erythrocytes. Washed PMNs, can be suspended in RPMI supplemented with 10% heat-inactivated fetal calf serum and mixed with ^{51}Cr labeled cells expressing CD20, at various ratios of effector cells to tumor cells (-effector cells:tumor cells). Purified human anti-CD20 IgGs can then be added at various concentrations. Irrelevant human IgG can be used as negative control. Assays can be carried out for 4 to 20 hours at 37 °C depending on the effector cell type used. Samples can be assayed for cytolysis by measuring ^{51}Cr release into the culture supernatant. Anti-CD20 monoclonal antibodies can also be tested in combinations with each other to determine whether cytolysis is enhanced with multiple monoclonal antibodies.

Human monoclonal antibodies which bind to CD20 also can be tested in an *in vivo* model (*e.g.*, in mice) to determine their efficacy in controlling growth of CD20-expressing tumor cells. These antibodies can be selected, for example, based on the following criteria, which are not intended to be exclusive:

1. binding to live cells expressing CD20;
2. low dissociation rate from CD20;
3. high affinity of binding to CD20;
4. binding to a unique epitope on CD20; and/or binding in a specific orientation to CD20, and/or binding to a specific form of CD20;
5. opsonization of cells expressing CD20;
6. mediation of growth inhibition, phagocytosis and/or killing of cells expressing CD20 in the presence of human effector cells;
7. ability to induce CDC on either CD55/CD59 negative or positive cells;
8. ability to induce homotypic adhesion;
9. ability to induce translocation into lipid rafts upon binding to CD20;
10. ability to induce apoptosis;
11. ability to induce ADCC on cells expressing CD20;
12. ability to deplete cells which express CD20; and/or
13. ability to deplete cells which express low levels of CD20 ($CD20^{low}$ cells).

Preferred human monoclonal antibodies of the invention meet one or more of these criteria.

Human monoclonal anti-CD20 antibodies can be tested for their ability to mediate CDC using a variety of known techniques. For example, serum for complement can be obtained from the blood of healthy subjects which can be centrifuged and harvested. To determine the CDC activity of various mAbs, different methods can be used. ^{51}Cr release can for example be measured or elevated membrane permeability can

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be assessed using a propidium iodide (PI) exclusion assay. Briefly, target cells can be washed and resuspended in RPMI-1% BSA at 1 x 10^6/ml. Various concentrations of mAb can be added to the cells and allowed to bind for 10-15 min at room temperature. Serum can then be added to a final concentration of 20% (v/v) and the cells incubated at 37 °C for 45 min. All cells from each sample can be added to the PI solution in a FACS tube. The mixture can then be assessed immediately by flow cytometry using a FACScalibur flow cytometer and analysed using CellQuest pro software (BD Biosciences, Mountain view, CA).

To test for the ability to initiate apoptosis, human monoclonal anti-CD20 antibodies can, for example, be incubated with CD20 positive tumor cells, *e.g.*, Daudi at 37 °C for about 20 hours. The cells can be harvested, washed in Annexin-V-FITC binding buffer (BD biosciences), and labeled with Annexin V-FITC (BD biosciences) for 15 min in the dark at 4 °C. All cells from each sample can be added to PI solution (10 µg/ml in PBS) in a FACS tube and assessed immediately by flow cytometry (as above).

In a particular embodiment of the invention, the human monoclonal antibodies are used in combination, *e.g.*, as a pharmaceutical composition comprising two or more anti-CD20 monoclonal antibodies. For example, human anti-CD20 monoclonal antibodies having different but complementary activities can be combined in a single therapy to achieve a desired therapeutic or diagnostic effect. In a preferred embodiment, the composition includes an anti-CD20 human monoclonal antibody that mediates CDC combined with another human anti-CD20 monoclonal antibody that induces apoptosis. In another embodiment, the composition includes an anti-CD20 human monoclonal antibody that mediates highly effective killing of target cells in the presence of effector cells, combined with another human anti-CD20 monoclonal antibody that inhibits the growth of cells expressing CD20.

II. Production of Transgenic Non-human Animals Which Generate Human Monoclonal Anti-CD20 Antibodies

In yet another aspect, the invention provides transgenic and transchromosomal non-human animals, such as transgenic or transchromosomal mice, which are capable of expressing human antibodies that specifically bind to CD20. In a particular embodiment, the invention provides a transgenic or transchromosomal mouse having a genome comprising a human heavy chain transgene, such that the mouse produces human anti- CD20 antibodies when immunized with cells expressing CD20. The human heavy chain transgene can be integrated into the chromosomal DNA of the mouse, as is the case for transgenic, *e.g.*, HuMAb mice, as described in detail herein and exemplified. Alternatively, the human heavy chain transgene can be maintained

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extrachromosomally, as is the case for transchromosomal (*e.g.*, KM) mice as described in WO 02/43478. Such transgenic and transchromosomal animals are capable of producing multiple isotypes of human monoclonal antibodies to CD20 (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J/V-J recombination and isotype switching. The design of a transgenic or transchromosomal non-human animal that responds to foreign antigen stimulation with a heterologous antibody repertoire, requires that the heterologous immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B cell development. This includes, for example, isotype switching of the heterologous heavy chain transgene. Accordingly, transgenes are constructed so as that isotype switching can be induced and one or more of the following characteristics of antibody genes: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

Not all of the foregoing criteria need be met. For example, in those embodiments wherein the endogenous immunoglobulin loci of the transgenic animal are functionally disrupted, the transgene need not activate allelic exclusion. Further, in those embodiments wherein the transgene comprises a functionally rearranged heavy and/or light chain immunoglobulin gene, the second criteria of functional gene rearrangement is unnecessary, at least for that transgene which is already rearranged. For background on molecular immunology, see, Fundamental Immunology, 2nd edition (1989), Paul William E., ed. Raven Press, N.Y.

In certain embodiments, the transgenic or transchromosomal non-human animals used to generate the human monoclonal antibodies of the invention contain rearranged, unrearranged or a combination of rearranged and unrearranged heterologous immunoglobulin heavy and light chain transgenes in the germline of the transgenic animal. Each of the heavy chain transgenes comprises at least one C_H gene. In addition, the heavy chain transgene may contain functional isotype switch sequences, which are capable of supporting isotype switching of a heterologous transgene encoding multiple C_H genes in the B cells of the transgenic animal. Such switch sequences may be those which occur naturally in the germline immunoglobulin locus from the species that serves as the source of the transgene C_H genes, or such switch sequences may be derived from those which occur in the species that is to receive the transgene construct (the transgenic animal). For example, a human transgene construct that is used to produce a transgenic mouse may produce a higher frequency of isotype switching events if it incorporates switch sequences similar to those that occur naturally in the mouse heavy chain locus, as presumably the mouse switch sequences are optimized to function with the mouse

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switch recombinase enzyme system, whereas the human switch sequences are not. Switch sequences may be isolated and cloned by conventional cloning methods, or may be synthesized *de novo* from overlapping synthetic oligonucleotides designed on the basis of published sequence information relating to immunoglobulin switch region sequences (Mills *et al.*, *Nucl. Acids Res.* 15:7305-7316 (1991); Sideras *et al.*, *Intl. Immunol.* 1:631-642 (1989)). For each of the foregoing transgenic animals, functionally rearranged heterologous heavy and light chain immunoglobulin transgenes are found in a significant fraction of the B cells of the transgenic animal (at least 10 percent).

The transgenes used to generate the transgenic non-human animals of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and at least one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of the transgenic non-human animal. In one aspect of the invention, the transgene is constructed such that the individual gene segments are unrearranged, *i.e.*, not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes support recombination of the V, D, and J gene segments (functional rearrangement) and preferably support incorporation of all or a portion of a D region gene segment in the resultant rearranged immunoglobulin heavy chain within the transgenic animal when exposed to CD20 antigen.

In an alternate embodiment, the transgenes comprise an unrearranged "mini-locus". Such transgenes typically comprise a substantial portion of the C, D, and J segments as well as a subset of the V gene segments. In such transgene constructs, the various regulatory sequences, *e.g.* promoters, enhancers, class switch regions, splice-donor and splice-acceptor sequences for RNA processing, recombination signals and the like, comprise corresponding sequences derived from the heterologous DNA. Such regulatory sequences may be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments may be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse. Alternatively, synthetic regulatory sequences may be incorporated into the transgene, wherein such synthetic regulatory sequences are not homologous to a functional DNA sequence that is known to occur naturally in the genomes of mammals. Synthetic regulatory sequences are designed according to consensus rules, such as, for example, those specifying the

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permissible sequences of a splice-acceptor site or a promoter/enhancer motif. For example, a minilocus comprises a portion of the genomic immunoglobulin locus having at least one internal (*i.e.*, not at a terminus of the portion) deletion of a non-essential DNA portion (*e.g.*, intervening sequence; intron or portion thereof) as compared to the naturally-occurring germline Ig locus.

Preferred transgenic and transchromosomal non-human animals, *e.g.*, mice, will exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a human after adjusting for volume.

The repertoire will ideally approximate that shown in a human when adjusted for volume, usually with a diversity at least about 10% as great, preferably 25 to 50% or more. Generally, at least about a thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending on the number of different V, J and D regions introduced into the mouse genome and driven by the additional diversity generated by V(-D-)J gene segment rearrangements random nucleotide additions at the joining regions. Typically, the immunoglobulins will exhibit an affinity (K_D) for preselected antigens of below 10^{-7} M, such as of below 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower.

Transgenic and transchromosomal non-human animals, *e.g.*, mice, as described above can be immunized with, for example, cells expressing CD20. Alternatively, the transgenic animals can be immunized with DNA encoding human CD20. The animals will then produce B cells which undergo class-switching via switch recombination (cis-switching) and express immunoglobulins reactive with CD20. The immunoglobulins can be human antibodies (also referred to as "human sequence antibodies"), wherein the heavy and light chain polypeptides are encoded by human transgene sequences, which may include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences; these human antibodies can be referred to as being substantially identical to a polypeptide sequence encoded by a human V_L and J_L or V_H, D_H and J_H gene segments, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. The variable regions of each antibody chain are typically at least 80 percent similar to human germline V, J, and, in the case of heavy chains, D, gene segments; frequently at least 85 percent similar to human germline sequences present on the transgene; often 90 or 95 percent or more similar to human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic mutation and VJ and VDJ joining, the human sequence antibodies will frequently have some variable region sequences which are not encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide

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positions) will cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

Another aspect of the invention includes B cells derived from transgenic or transchromosomal non-human animals as described herein. The B cells can be used to generate hybridomas expressing human monoclonal antibodies which bind with high affinity (*e.g.*, a dissociation equilibrium constant (K_D) of lower than 10^{-7} M) to human CD20. Thus, in another embodiment, the invention provides a hybridoma which produces a human antibody having an affinity (K_D) of below 10^{-7} M, such as of below 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by scatchard analysis of CD20 expressing cells using a radio-actively labeled monoclonal antibody or by determination of the half-maximal binding concentration using FACS analysis.

Herein the monoclonal antibody comprises a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, a D region, and a human J_H segment, and (2) a constant region encoded by a human C_H gene segment.

The development of high affinity human monoclonal antibodies against CD20 can be facilitated by a method for expanding the repertoire of human variable region gene segments in a transgenic non-human animal having a genome comprising an integrated human immunoglobulin transgene, said method comprising introducing into the genome a V gene transgene comprising V region gene segments which are not present in said integrated human immunoglobulin transgene. Often, the V region transgene is a yeast artificial chromosome comprising a portion of a human V_H or V_L (V_K) gene segment array, as may naturally occur in a human genome or as may be spliced together separately by recombinant methods, which may include out-of-order or omitted V gene segments. Often at least five or more functional V gene segments are contained on the YAC. In this variation, it is possible to make a transgenic animal produced by the V repertoire expansion method, wherein the animal expresses an immunoglobulin chain comprising a variable region sequence encoded by a V region gene segment present on the V region transgene and a C region encoded on the human Ig transgene. By means of the V repertoire expansion method, transgenic animals having at least 5 distinct V genes can be generated; as can animals containing at least about 24 V genes or more. Some V gene segments may be non-functional (*e.g.*,

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pseudogenes and the like); these segments may be retained or may be selectively deleted by recombinant methods available to the skilled artisan, if desired.

Once the mouse germline has been engineered to contain a functional YAC having an expanded V segment repertoire, substantially not present in the human Ig transgene containing the J and C gene segments, the trait can be propagated and bred into other genetic backgrounds, including backgrounds where the functional YAC having an expanded V segment repertoire is bred into a non-human animal germline having a different human Ig transgene. Multiple functional YACs having an expanded V segment repertoire may be bred into a germline to work with a human Ig transgene (or multiple human Ig transgenes). Although referred to herein as YAC transgenes, such transgenes when integrated into the genome may substantially lack yeast sequences, such as sequences required for autonomous replication in yeast; such sequences may optionally be removed by genetic engineering (*e.g.*, restriction digestion and pulsed-field gel electrophoresis or other suitable method) after replication in yeast is no longer necessary (*i.e.*, prior to introduction into a mouse ES cell or mouse prozygote). Methods of propagating the trait of human sequence immunoglobulin expression, include breeding a transgenic animal having the human Ig transgene(s), and optionally also having a functional YAC having an expanded V segment repertoire. Both V_H and V_L gene segments may be present on the YAC. The transgenic animal may be bred into any background desired by the practitioner, including backgrounds harboring other human transgenes, including human Ig transgenes and/or transgenes encoding other human lymphocyte proteins. The invention also provides a high affinity human sequence immunoglobulin produced by a transgenic mouse having an expanded V region repertoire YAC transgene. Although the foregoing describes a preferred embodiment of the transgenic animal of the invention, other embodiments are contemplated which have been classified in three categories:

I. Transgenic animals containing an unrearranged heavy and rearranged light chain immunoglobulin transgene;

II. Transgenic animals containing an unrearranged heavy and unrearranged light chain immunoglobulin transgene; and

III. Transgenic animal containing rearranged heavy and an unrearranged light chain immunoglobulin transgene.

Of these categories of transgenic animal, the preferred order of preference is as follows II > I > III where the endogenous light chain genes (or at least the K gene) have been knocked out by homologous recombination (or other method) and I > II > III where the endogenous light chain genes have not been knocked out and must be dominated by allelic exclusion.

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III. Bispecific/ Multispecific Molecules Which Bind to CD20

In yet another embodiment of the invention, human monoclonal antibodies to CD20 can be derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, an Fab' fragment) to generate a bispecific or multispecific molecule which binds to multiple binding sites or target epitopes. For example, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, peptide or binding mimetic.

Accordingly, the present invention includes bispecific and multispecific molecules comprising at least one first binding specificity for CD20 and a second binding specificity for a second target epitope. In a particular embodiment of the invention, the second target epitope is an Fc receptor, *e.g.*, human FcγRI (CD64) or a human Fcα receptor (CD89), or a T cell receptor, *e.g.*, CD3. Therefore, the invention includes bispecific and multispecific molecules capable of binding both to FcγR, FcαR or FcεR expressing effector cells (*e.g.*, monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing CD20. These bispecific and multispecific molecules target CD20 expressing cells to effector cell and, like the human monoclonal antibodies of the invention, trigger Fc receptor-mediated effector cell activities, such as phagocytosis of a CD20 expressing cells, antibody dependent cellular cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

Bispecific and multispecific molecules of the invention can further include a third binding specificity, in addition to an anti-Fc binding specificity and an anti-CD20 binding specificity. In one embodiment, the third binding specificity is an anti-enhancement factor (EF) portion, *e.g.*, a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, *e.g.*, an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the F_c receptor or target cell antigen. The "anti-enhancement factor portion" can bind an F_c receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T cell (*e.g.*, via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell that results in an increased immune response against the target cell).

In one embodiment, the bispecific and multispecific molecules of the invention comprise as a binding specificity at least one antibody, including, *e.g.*, an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as

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described in Ladner *et al.* US 4,946,778. The antibody may also be a binding-domain immunoglobulin fusion protein as disclosed in US 2003/0118592 and US 2003/0133939.

In one embodiment bispecific and multispecific molecules of the invention comprise a binding specificity for an FcγR or an FcαR present on the surface of an effector cell, and a second binding specificity for a target cell antigen, *e.g.*, CD20.

In one embodiment, the binding specificity for an Fc receptor is provided by a human monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ-chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), FcγRII(CD32), and FcγRIII (CD16). In one preferred embodiment, the Fcγ receptor is a human high affinity FcγRI.

The production and characterization of these preferred monoclonal antibodies are described by Fanger *et al.* in WO 88/00052 and in US 4,954,617. These antibodies bind to an epitope of FcγRI, FcγRII or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. In other embodiments, the anti-Fcγ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and characterization of the H22 antibody is described in Graziano, R.F. *et al.* (1995) J. Immunol 155 (10): 4996-5002 and WO 94/10332. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession No. CRL 11177.

In still other preferred embodiments, the binding specificity for an Fc receptor is provided by an antibody that binds to a human IgA receptor, *e.g.*, an Fc-alpha receptor (FcαRI (CD89)), the binding of which is preferably not blocked by human immunoglobulin A (IgA). The term "IgA receptor" is intended to include the gene product of one α-gene (FcαRI) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcαRI (CD89) is constitutively expressed on monocytes/macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαRI has medium affinity for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton, H.C. *et al.* (1996) Critical Reviews in Immunology 16:423-440). Four FcαRI-specific monoclonal antibodies, identified as A3, A59, A62 and A77, which bind FcαRI outside the IgA ligand binding domain, have been described (Monteiro, R.C. *et al.* (1992) *J. Immunol.* 148:1764).

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FcαRI and FcγRI are preferred trigger receptors for use in the invention because they are (1) expressed primarily on immune effector cells, *e.g.*, monocytes, PMNs, macrophages and dendritic cells; (2) expressed at high levels (*e.g.*, 5,000-100,000 per cell); (3) mediators of cytotoxic activities (*e.g.*, ADCC, phagocytosis); (4) mediate enhanced antigen presentation of antigens, including self-antigens, targeted to them.

In another embodiment the bispecific molecule is comprised by two human monoclonal antibodies according to the invention which have complementary functional activities, such as one antibody predominately working by inducing CDC and the other antibody predominately working by inducing apoptosis, *e.g.*, 2F2 in combination with 11B8.

In other embodiments, bispecific and multispecific molecules of the invention further comprise a binding specificity which recognizes, *e.g.*, binds to, a target cell antigen, *e.g.*, CD20. In a preferred embodiment, the binding specificity is provided by a human monoclonal antibody of the present invention.

An "effector cell specific antibody" as used herein refers to an antibody or functional antibody fragment that binds the Fc receptor of effector cells. Preferred antibodies for use in the subject invention bind the Fc receptor of effector cells at a site which is not bound by endogenous immunoglobulin.

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, *e.g.*, lymphocytes (*e.g.*, B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and basophils. Some effector cells express specific Fc receptors and carry out specific immune functions. In preferred embodiments, an effector cell is capable of inducing antibody-dependent cellular cytotoxicity (ADCC), *e.g.*, a neutrophil capable of inducing ADCC. For example, monocytes, macrophages, which express FcR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens. In other embodiments, an effector cell can phagocytose a target antigen, target cell, or microorganism. The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon gamma (IFN-γ). This enhanced expression increases the cytotoxic activity of FcγRI-bearing cells against targets. An effector cell can phagocytose or lyse a target antigen or a target cell.

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"Target cell" shall mean any undesirable cell in a subject (*e.g.*, a human or animal) that can be targeted by a composition (*e.g.*, a human monoclonal antibody, a bispecific or a multispecific molecule) of the invention. In preferred embodiments, the target cell is a cell expressing or overexpressing CD20. Cells expressing CD20 typically include B cells and B cell tumors.

While human monoclonal antibodies are preferred, other antibodies which can be employed in the bispecific or multispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies. Such murine, chimeric and humanized monoclonal antibodies can be prepared by methods known in the art.

Bispecific and multispecific molecules of the present invention can be made using chemical techniques (see *e.g.*, D. M. Kranz *et al.* (1981) *Proc. Natl. Acad. Sci.* USA 78:5807), "polydoma" techniques (See US 4,474,893, to Reading), or recombinant DNA techniques.

In particular, bispecific and multispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, *e.g.*, the anti-FcR and anti-CD20 binding specificities, using methods known in the art and described in the examples provided herein. For example, each binding specificity of the bispecific and multispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (see *e.g.*, Karpovsky *et al.* (1984) *J. Exp. Med.* 160:1686; Liu, MA *et al.* (1985) *Proc. Natl. Acad. Sci.* USA 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan *et al.* (*Science* (1985) 229:81-83), and Glennie *et al.* (*J. Immunol.* (1987) 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific and multispecific molecule is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A bispecific and multispecific molecule of the invention, *e.g.*, a bispecific molecule can be a single chain molecule, such as a single

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chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific and multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- and multispecific molecules are described for example in US 5,260,203; US 5,455,030; US 4,881,175; US 5,132,405; US 5,091,513; US 5,476,786; US 5,013,653; US 5,258,498; and US 5,482,858.

Binding of the bispecific and multispecific molecules to their specific targets can be confirmed by enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), FACS analysis, a bioassay (*e.g.*, growth inhibition), or a Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using *e.g.*, an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

IV. Immunoconjugates

In another aspect, the present invention features a human anti-CD20 monoclonal antibody conjugated to a therapeutic moiety, such as a cytotoxin, a drug (*e.g.*, an immunosuppressant) or a radioisotope. Such conjugates are referred to herein as "immunoconjugates". Immunoconjugates which include one or more cytotoxins are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (*e.g.*, kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof.

Suitable therapeutic agents for forming immunoconjugates of the invention include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, fludarabin, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine

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(BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine). In a preferred embodiment, the therapeutic agent is a cytotoxic agent or a radiotoxic agent. In another embodiment, the therapeutic agent is an immunosuppressant. In yet another embodiment, the therapeutic agent is GM-CSF. In a preferred embodiment, the therapeutic agent is doxorubicin, cisplatin, bleomycin, sulfate, carmustine, chlorambucil, cyclophosphamide or ricin A.

Antibodies of the present invention also can be conjugated to a radioisotope, *e.g.*, iodine-131, yttrium-90 or indium-111, to generate cytotoxic radiopharmaceuticals for treating a CD20-related disorder, such as a cancer. The antibody conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-γ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

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In a further embodiment, the human monoclonal antibodies according to the invention are attached to a linker-chelator, *e.g.*, tiuxetan, which allows for the antibody to be conjugated to a radioisotope.

V. Pharmaceutical Compositions

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of human monoclonal antibodies of the present invention. The pharmaceutical compositions may be formulated with pharmaceutically acceptable carriers or diluents as well as any other known adjuvants and excipients in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. In one embodiment, the compositions include a combination of multiple (*e.g.*, two or more) isolated human antibodies of the invention which act by different mechanisms, *e.g.*, one antibody which predominately acts by inducing CDC in combination with another antibody which predominately acts by inducing apoptosis.

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-inflammatory agent or at least one immunosuppressive agent. In one embodiment such therapeutic agents include one or more anti-inflammatory agents, such as a steroidal drug or a NSAID (nonsteroidal anti-inflammatory drug). Preferred agents include, for example, aspirin and other salicylates, Cox-2 inhibitors, such as rofecoxib (Vioxx) and celecoxib (Celebrex), NSAIDs such as ibuprofen (Motrin, Advil), fenoprofen (Nalfon), naproxen (Naprosyn), sulindac (Clinoril), diclofenac (Voltaren), piroxicam (Feldene), ketoprofen (Orudis), diflunisal (Dolobid), nabumetone (Relafen), etodolac (Lodine), oxaprozin (Daypro), and indomethacin (Indocin).

In another embodiment, such therapeutic agents include one or more DMARDs, such as methotrexate (Rheumatrex), hydroxychloroquine (Plaquenil), sulfasalazine (Asulfidine), pyrimidine synthesis inhibitors, *e.g.*, leflunomide (Arava), IL-1 receptor blocking agents, *e.g.*, anakinra (Kineret), and TNF-α blocking agents, *e.g.*, etanercept (Enbrel), infliximab (Remicade) and adalimumab.

In another embodiment, such therapeutic agents include one or more immunosuppressive agents, such as cyclosporine (Sandimmune, Neoral) and azathioprine (Imural).

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In yet another embodiment, such therapeutic agents include one or more chemotherapeutics, such as doxorubicin (Adriamycin), cisplatin (Platinol), bleomycin (Blenoxane), carmustine (Gliadel), cyclophosphamide (Cytoxan, Procytox, Neosar), and chlorambucil (Leukeran).

In another embodiment, human antibodies of the present invention may be administered in combination with chlorambucil and prednisolone; cyclophosphamide and prednisolone; cyclophosphamide, vincristine, and prednisone; cyclophosphamide, vincristine, doxorubicin, and prednisone; fludarabine and anthracycline; or in combination with other common multi-drugs regimens for NHL, such as disclosed, *e.g.*, in Non-Hodgkin's Lymphomas: Making sense of Diagnosis, Treatment, and Options, Lorraine Johnston, 1999, O'Reilly and Associates, Inc.

In yet another embodiment, the human antibodies may be administered in conjunction with radiotherapy and/or autologous peripheral stem cell or bone marrow transplantation.

In still another embodiment, the human antibodies may be administered in combination with one or more antibodies selected from anti-CD25 antibodies, anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, anti-CD38 antibodies, anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies (*e.g.*, efalizumab), anti-alpha-4/beta-1 integrin (VLA4) antibodies (*e.g.*, natalizumab), and CTLA4-Ig.

In a particular embodiment, the human monoclonal antibodies are administered in combination with an anti-CD25 antibody for the treatment of bullous pemphigoid, *e.g.*, in patients with graft-versus-host disease.

In another particular embodiment, the human monoclonal antibodies are administered in combination with one or more antibodies selected from anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, and anti-CD38 antibodies for the treatment of malignant diseases.

In still another particular embodiment, the human antibodies are administered in combination with one or more antibodies selected from anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies (*e.g.*, efalizumab), anti-alpha-4/beta-1 integrin (VLA4) antibodies (*e.g* natalizumab), and CTLA4-Ig for the treatment of inflammatory diseases.

In yet a further embodiment, the human antibodies may be administered in combination with an anti-C3b(i) antibody in order to enhance complement activation.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably,

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the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) J. Pharm. Sci. 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for the preparation of such formulations are generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neuroimmunol.* 7:27).

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Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical

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carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

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Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In one embodiment the human monoclonal antibodies of the invention are administered in crystalline form by subcutaneous injection, cf. Yang *et al.* (2003) *PNAS*, 100(12):6934-6939

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (more preferably, 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in

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combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

In one embodiment, the human monoclonal antibodies according to the invention may be administered by infusion in a weekly dosage of 10 to 500 mg/m^2, such as 200 to 400 mg/m^2. Such administration may be repeated, *e.g.*, 1 to 8 times, such as 3 to 5 times. The administration may be performed by continuous infusion over a period of from 2 to 24 hours, such as of from 2 to 12 hours.

In another embodiment, the human monoclonal antibodies are administered by slow continuous infusion over a long period, such as more than 24 hours, in order to reduce toxic side effects.

In still another embodiment the human monoclonal antibodies are administered in a weekly dosage of from 250 mg to 2000 mg, such as for example 300 mg, 500 mg, 700 mg, 1000 mg, 1500 mg or 2000 mg, for up to 8 times, such as from 4 to 6 times. The administration may be performed by continuous infusion over a period of from 2 to 24 hours, such as of from 2 to 12 hours. Such regimen may be repeated one or more times as necessary, for example, after 6 months or 12 months. The dosage can be determined or adjusted by measuring the amount of circulating monoclonal anti-CD20 antibodies upon administration in a biological sample by using anti-idiotypic antibodies which target the anti-CD20 antibodies.

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In yet another embodiment, the human monoclonal antibodies are administered by maintenance therapy, such as, *e.g.*, once a week for a period of 6 months or more.

In still another embodiment, the human monoclonal antibodies according to the invention may be administered by a regimen including one infusion of a human monoclonal antibody against CD20 followed by an infusion of a human monoclonal antibody against CD20 conjugated to a radioisotope. The regimen may be repeated, *e.g.*, 7 to 9 days later.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in US 5,399,163; US 5,383,851; US 5,312,335; US 5,064,413; US 4,941,880; US 4,790,824; or US 4,596,556. Examples of well-known implants and modules useful in the present invention include: US 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; US 4,486,194, which discloses a therapeutic device for administering medicants through the skin; US 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; US 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; US 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and US 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the human monoclonal antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, US 4,522,811; US 5,374,548; and US 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*see, e.g.*, V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, US 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L.

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Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the desired area, *e.g.*, the site of inflammation or infection, or the site of a tumor. The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

In a further embodiment, human monoclonal antibodies of the invention can be formulated to prevent or reduce their transport across the placenta. This can be done by methods known in the art, *e.g.*, by PEGylation of the antibodies or by use of F(ab)2' fragments. Further references can be made to "Cunningham-Rundles C, Zhuo Z, Griffith B, Keenan J. (1992) Biological activities of polyethylene-glycol immunoglobulin conjugates. Resistance to enzymatic degradation. *J Immunol Methods.* 152:177-190; and to "Landor M. (1995) Maternal-fetal transfer of immunoglobulins, *Ann Allergy Asthma Immunol* 74:279-283. This is particularly relevant when the antibodies are used for treating or preventing recurrent spontaneous abortion.

A "therapeutically effective dosage" for tumor therapy can be measured by objective tumor responses which can either be complete or partial. A complete response (CR) is defined as no clinical, radiological or other evidence of disease. A partial response (PR) results from a reduction in aggregate tumor size of greater than 50%. Median time to progression is a measure that characterizes the durability of the objective tumor response.

A "therapeutically effective dosage" for tumor therapy can also be measured by its ability to stabilize the progression of disease. The ability of a compound to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit cell growth or apoptosis by *in vitro* assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

A "therapeutically effective dosage" for rheumatoid arthritis preferably will result in an ACR20 Preliminary Definition of Improvement in the patients, more preferred in an ACR50 Preliminary Definition of Improvement and even more preferred in an ARC70 Preliminary Definition of Improvement.

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ACR20 Preliminary Definition of Improvement is defined as: ≥20% improvement in: Tender Joint Count (TCJ) and Swollen Joint Count (SWJ) and ≥ 20% improvement in 3 of following 5 assessments: Patient Pain Assessment (VAS), Patient Global assessment (VAS), Physician Global Assessment (VAS), Patent Self-Assessed Disability (HAQ), Acute Phase Reactant (CRP or ESR).

ACR50 and ACR70 are defined in the same way with ≥50% and ≥70% improvements, respectively. For further details see Felson *et al.* in American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis; Arthritis Rheumatism (1995) 38: 727-735.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

VI. Uses and Methods of the Invention

The human antibodies (including immunoconjugates, bispecifics/multispecifics, compositions and other derivatives described herein) of the present invention have numerous *in vitro* and *in vivo* diagnostic and therapeutic utilities involving the diagnosis and treatment of disorders involving cells expressing CD20. For example, the antibodies can be administered to cells in culture, *e.g.*, *in vitro* or *ex vivo*, or to human subjects, *e.g.*, *in vivo*, to treat, prevent and to diagnose a variety of disorders. As used herein, the term "subject" is intended to include human and non-human animals which respond to the human antibodies against CD20. Preferred subjects include human patients having disorders that can be corrected or ameliorated by inhibiting or controlling B cells (normal or malignant).

For example, in one embodiment, human antibodies of the present invention can be used to treat a subject with a tumorigenic disorder, *e.g.*, a disorder characterized by the presence of tumor cells expressing CD20 including, for example, B cell lymphoma, *e.g.*, NHL. Examples of tumorigenic diseases which can be treated

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and/or prevented include B cell lymphoma, *e.g.*, NHL, including precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia(CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), including low-grade, intermediate-grade and high-grade FL, cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL).

Further examples of B cell non-Hodgkin's lymphomas are lymphomatoid granulomatosis, primary effusion lymphoma, intravascular large B cell lymphoma, mediastinal large B cell lymphoma, heavy chain diseases (including γ, μ, and α disease), lymphomas induced by therapy with immunosuppressive agents, such as cyclosporine-induced lymphoma, and methotrexate-induced lymphoma.

In a further embodiment, the human antibodies of the present invention can be used to treat Hodgkin's lymphoma.

Examples of immune disorders in which CD20 expressing B cells are involved which can be treated and/or prevented include autoimmune disorders, such as psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases. Further examples are severe acute respiratory distress syndrome and choreoretinitis. Furthermore, other diseases and disorders include those caused by or mediated by infection of B-cells with virus, such as Epstein-Barr virus (EBV).

Further examples of inflammatory, immune and/or autoimmune disorders in which autoantibodies and/or excessive B lymphocyte activity are prominent and which can be treated and/or prevented, include the following:

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vasculitides and other vessel disorders, such as microscopic polyangiitis, Churg-Strauss syndrome, and other ANCA-associated vasculitides, polyarteritis nodosa, essential cryoglobulinaemic vasculitis, cutaneous leukocytoclastic angiitis, Kawasaki disease, Takayasu arteritis, giant cell arthritis, Henoch-Schönlein purpura, primary or isolated cerebral angiitis, erythema nodosum, thrombangiitis obliterans, thrombotic thrombocytopenic purpura (including hemolytic uremic syndrome), and secondary vasculitides, including cutaneous leukocytoclastic vasculitis (*e.g.*, secondary to hepatitis B, hepatitis C, Waldenström's macroglobulinemia, B-cell neoplasias, rheumatoid arthritis, Sjögren's syndrome, or systemic lupus erythematosus); further examples are erythema nodosum, allergic vasculitis, panniculitis, Weber-Christian disease, purpura hyperglobulinaemica, and Buerger's disease;

skin disorders, such as contact dermatitis, linear IgA dermatosis, vitiligo, pyoderma gangrenosum, epidermolysis bullosa acquisita, pemphigus vulgaris (including cicatricial pemphigoid and bullous pemphigoid), alopecia areata (including alopecia universalis and alopecia totalis), dermatitis herpetiformis, erythema multiforme, and chronic autoimmune urticaria (including angioneurotic edema and urticarial vasculitis);

immune-mediated cytopenias, such as autoimmune neutropenia, and pure red cell aplasia;

connective tissue disorders, such as CNS lupus, discoid lupus erythematosus, CREST syndrome, mixed connective tissue disease, polymyositis/dermatomyositis, inclusion body myositis, secondary amyloidosis, cryoglobulinemia type I and type II, fibromyalgia, phospholipid antibody syndrome, secondary hemophilia, relapsing polychondritis, sarcoidosis, stiff man syndrome, and rheumatic fever; a further example is eosinophil fasciitis;

arthritides, such as ankylosing spondylitis, juvenile chronic arthritis, adult Still's disease, and SAPHO syndrome; further examples are sacroileitis, reactive arthritis, Still's disease, and gout;

hematologic disorders, such as aplastic anemia, primary hemolytic anemia (including cold agglutinin syndrome), hemolytic anemia secondary to CLL or systemic lupus erythematosus; POEMS syndrome, pernicious anemia, and Waldemström's purpura hyperglobulinaemica; further examples are agranulocytosis, autoimmune neutropenia, Franklin's disease, Seligmann's disease, μ-chain disease, paraneoplastic syndrome secondary to thymoma and lymphomas, and factor VIII inhibitor formation;

endocrinopathies, such as polyendocrinopathy, and Addison's disease; further examples are autoimmune hypoglycemia, autoimmune hypothyroidism, autoimmune insulin syndrome, de Quervain's thyroiditis, and insulin receptor antibody-mediated insulin resistance;

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hepato-gastrointestinal disorders, such as celiac disease, Whipple's disease, primary biliary cirrhosis, chronic active hepatitis, and primary sclerosing cholangiitis; a further example is autoimmune gastritis;

nephropathies, such as rapid progressive glomerulonephritis, post-streptococcal nephritis, Goodpasture's syndrome, membranous glomerulonephritis, and cryoglobulinemic nephritis; a further example is minimal change disease;

neurological disorders, such as autoimmune neuropathies, mononeuritis multiplex, Lambert-Eaton's myasthenic syndrome, Sydenham's chorea, tabes dorsalis, and Guillain-Barré's syndrome; further examples are myelopathy/tropical spastic paraparesis, myasthenia gravis, acute inflammatory demyelinating polyneuropathy, and chronic inflammatory demyelinating polyneuropathy;

cardiac and pulmonary disorders, such as fibrosing alveolitis, bronchiolitis obliterans, allergic aspergillosis, cystic fibrosis, Löffler's syndrome, myocarditis, and pericarditis; further examples are hypersensitivity pneumonitis, and paraneoplastic syndrome secondary to lung cancer;

allergic disorders, such as bronchial asthma and hyper-IgE syndrome; a further example is amaurosis fugax;

ophthalmologic disorders, such as idiopathic chorioretinitis;

infectious diseases, such as parvovirus B infection (including hands-and-socks syndrome); and

gynecological-obstretical disorders, such as recurrent abortion, recurrent fetal loss, and intrauterine growth retardation; a further example is paraneoplastic syndrome secondary to gynaecological neoplasms;

male reproductive disorders, such as paraneoplastic syndrome secondary to testicular neoplasms; and

transplantation-derived disorders, such as allograft and xenograft rejection, and graft-versus-host disease.

In one embodiment, the disease is an inflammatory, immune and/or autoimmune disorder selected from ulcerative colitis, Crohn's disease, juvenile onset diabetes, multiple sclerosis, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia (including autoimmune hemolytic anemia), myasthenia gravis, systemic sclerosis, and pemphigus vulgaris.

In another embodiment, human antibodies of the invention can be used to detect levels of CD20, or levels of cells which contain CD20 on their membrane surface, which levels can then be linked to certain disease symptoms. Alternatively, the antibodies can be used to deplete or interact with the function of CD20 expressing cells, thereby implicating these cells as important mediators of the disease. This can be

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achieved by contacting a sample and a control sample with the anti-CD20 antibody under conditions that allow for the formation of a complex between the antibody and CD20. Any complexes formed between the antibody and CD20 are detected and compared in the sample and the control.

Human antibodies of the invention can be initially tested for binding activity associated with therapeutic or diagnostic use *in vitro*. For example, the antibodies can be tested using flow cytometric assays described in the Examples below. Moreover, activity of the antibodies in triggering at least one effector-mediated effector cell activity, including inhibiting the growth of and/or killing of cells expressing CD20, can be assayed. For example, the ability of the antibodies to trigger CDC and/or apoptosis can be assayed. Protocols for assaying for CDC, homotypic adhesion, molecular clustering or apoptosis are described in the Examples below.

Human antibodies of the invention also have additional utility in therapy and diagnosis of a variety of CD20-related diseases. For example, the human antibodies can be used to elicit *in vivo* or *in vitro* one or more of the following biological activities: to inhibit the growth of and/or differentiation of a cell expressing CD20; to kill a cell expressing CD20; to mediate phagocytosis or ADCC of a cell expressing CD20 in the presence of human effector cells; to mediate CDC of a cell expressing CD20 in the presence of complement; to mediate apoptosis of a cell expressing CD20; to induce homotypic adhesion; and/or to induce translocation into lipid rafts upon binding CD20.

In a particular embodiment, the human antibodies are used *in vivo* to treat, prevent or diagnose a variety of CD20-related diseases. Examples of CD20-related diseases include, among others, B cell lymphoma, *e.g.*, NHL, and immune diseases, *e.g.*, autoimmune diseases, such as those listed above.

In a particular embodiment, the antibodies of the invention are used to treat or to prevent NHL, as the antibodies deplete the CD20 bearing tumor cells).

Non-Hodgkin's lymphoma is a type of B cell lymphoma. Lymphomas, *e.g.*, B cell lymphomas, are a group of related cancers that arise when a lymphocyte (a blood cell) becomes malignant. The normal function of lymphocytes is to defend the body against invaders: germs, viruses, fungi, even cancer. There are many subtypes and maturation stages of lymphocytes and, therefore, there are many kinds of lymphomas. Like normal cells, malignant lymphocytes can move to many parts of the body. Typically, lymphoma cells form tumors in the lymphatic system: bone marrow, lymph nodes, spleen, and blood. However, these cells can migrate to other organs. Certain types of lymphoma will tend to grow in locations in which the normal version of the cell resides. For example, it's common for follicular NHL tumors to develop in the lymph nodes.

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CD20 is usually expressed at elevated levels on neoplastic (*i.e.*, tumorigenic) B cells associated with NHL. Accordingly, CD20 binding antibodies of the invention can be used to deplete CD20 bearing tumor cells which lead to NHL and, thus, can be used to prevent or treat this disease.

Human antibodies (*e.g.*, human monoclonal antibodies, multispecific and bispecific molecules) of the present invention also can be used to block or inhibit other effects of CD20. For example, it is known that CD20 is expressed on B lymphocytes and is involved in the proliferation and/or differentiation of these cells. Since B lymphocytes function as immunomodulators, CD20 is an important target for antibody mediated therapy to target B lymphocytes, *e.g.*, to inactivate or kill B lymphocytes, involved in autoimmune disorders. Such autoimmune disorders include, for example, the above listed diseases

Suitable routes of administering the antibody compositions (*e.g.*, human monoclonal antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition. Furthermore, tumor load can be determined and used to calculate suitable dosages.

As previously described, human anti-CD20 antibodies of the invention can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The antibody can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, an anti-cancer therapy, *e.g.*, radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide. Co-administration of the human anti-CD20 antibodies of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the antibody.

Target-specific effector cells, *e.g.*, effector cells linked to compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be used as therapeutic agents. Effector cells for targeting can be human leukocytes such as macrophages, neutrophils or monocytes. Other cells include eosinophils, natural

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killer cells and other IgG- or IgA-receptor bearing cells. If desired, effector cells can be obtained from the subject to be treated. The target-specific effector cells, can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells administered can be in the order of 10^8 to 10^9 but will vary depending on the therapeutic purpose. In general, the amount will be sufficient to obtain localization at the target cell, *e.g.*, a tumor cell expressing CD20, and to effect cell killing by, *e.g.*, phagocytosis. Routes of administration can also vary.

Therapy with target-specific effector cells can be performed in conjunction with other techniques for removal of targeted cells. For example, anti-tumor therapy using the compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention and/or effector cells armed with these compositions can be used in conjunction with chemotherapy. Additionally, combination immunotherapy may be used to direct two distinct cytotoxic effector populations toward tumor cell rejection. For example, anti-CD20 antibodies linked to anti-Fc-γRI or anti-CD3 may be used in conjunction with IgG- or IgA-receptor specific binding agents.

Bispecific and multispecific molecules of the invention can also be used to modulate FcγR or FcαR levels on effector cells, such as by capping and elimination of receptors on the cell surface. Mixtures of anti-Fc receptors can also be used for this purpose.

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention which have complement binding sites, such as portions from IgG1, -2, or -3 or IgM which bind complement, can also be used in the presence of complement. In one embodiment, *ex vivo* treatment of a population of cells comprising target cells with a binding agent of the invention and appropriate effector cells can be supplemented by the addition of complement or serum containing complement. Phagocytosis of target cells coated with a binding agent of the invention can be improved by binding of complement proteins. In another embodiment target cells coated with the compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be lysed by complement. In yet another embodiment, the compositions of the invention do not activate complement.

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention can also be administered together with complement. Accordingly, within the scope of the invention are compositions comprising human antibodies, multispecific or bispecific molecules and serum or complement. These compositions are advantageous in that the complement is located in close proximity to the human antibodies, multispecific or bispecific molecules. Alternatively, the human antibodies, multispecific or bispecific molecules of the invention and the complement or serum can be administered separately. Binding of the

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compositions of the present invention to target cells causes translocation of the CD20 antigen-antibody complex into lipid rafts of the cell membrane. Such translocation creates a high density of antigen-antibody complexes which may efficiently activate and/or enhance CDC.

Also within the scope of the present invention are kits comprising the antibody compositions of the invention (*e.g.*, human antibodies and immunoconjugates) and instructions for use. The kit can further contain one or more additional reagents, such as an immunosuppressive reagent, a cytotoxic agent or a radiotoxic agent, or one or more additional human antibodies of the invention (*e.g.*, a human antibody having a complementary activity).

Accordingly, patients treated with antibody compositions of the invention can be additionally administered (prior to, simultaneously with, or following administration of a human antibody of the invention) with another therapeutic agent, such as a cytotoxic or radiotoxic agent, which enhances or augments the therapeutic effect of the human antibodies.

In other embodiments, the subject can be additionally treated with an agent that modulates, *e.g.*, enhances or inhibits, the expression or activity of Fcγ or Fcα receptors by, for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the multispecific molecule include of granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF).

The compositions (*e.g.*, human antibodies, multispecific and bispecific molecules) of the invention can also be used to target cells expressing FcγR or CD20, for example for labeling such cells. For such use, the binding agent can be linked to a molecule that can be detected. Thus, the invention provides methods for localizing *ex vivo* or *in vitro* cells expressing Fc receptors, such as FcγR, or CD20. The detectable label can be, *e.g.*, a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor.

In a particular embodiment, the invention provides methods for detecting the presence of CD20 antigen in a sample, or measuring the amount of CD20 antigen, comprising contacting the sample, and a control sample, with a human monoclonal antibody which specifically binds to CD20, under conditions that allow for formation of a complex between the antibody or portion thereof and CD20. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of CD20 antigen in the sample.

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In other embodiments, the invention provides methods for treating a disorder involving cells expressing CD20 in a subject, *e.g.*, non-Hodgkin's lymphoma or rheumatoid arthritis, by administering to the subject the human antibodies described above. Such antibodies and derivatives thereof are used to inhibit CD20 induced activities associated with certain disorders, *e.g.*, proliferation and/or differentiation. By contacting the antibody with CD20 (*e.g.*, by administering the antibody to a subject), the ability of CD20 to induce such activities is inhibited and, thus, the associated disorder is treated.

Accordingly, in another embodiment, the present invention provides a method for treating or preventing a tumorigenic disorder involving CD20 expressing cells, *e.g.*, NHL. The method involves administering to a subject an antibody composition of the present invention in an amount effective to treat or prevent the disorder. The antibody composition can be administered alone or along with another therapeutic agent, such as a cytotoxic or a radiotoxic agent which acts in conjunction with or synergistically with the antibody composition to treat or prevent the diseases involving CD20 expressing cells. In a particularly preferred embodiment, the present invention provides a method for treating non-Hodgkin's lymphoma.

In another embodiment, the present invention provides a method for treating or preventing an autoimmune disorder involving human CD20 expressing cells, *e.g.*, those diseases as listed above. The method involves administering to a subject an antibody composition of the present invention in an amount effective to treat or prevent the disorder. The antibody composition can be administered alone or along with another therapeutic agent, such as an immunosuppressant which acts in conjunction with or synergistically with the antibody composition to treat or prevent the disease involving cells expressing CD20.

In still another embodiment, the invention provides a method for detecting the presence or quantifying the amount of CD20-expressing cells *in vivo* or *in vitro*. The method comprises (i) administering to a subject a composition (*e.g.*, a multi- or bispecific molecule) of the invention conjugated to a detectable marker; (ii) exposing the subject to a means for detecting said detectable marker to identify areas containing CD20-expressing cells.

In yet another embodiment; immunoconjugates of the invention can be used to target compounds (*e.g.*, therapeutic agents, labels, cytotoxins, radiotoxins immunosuppressants, *etc.*) to cells which have CD20 expressed on their surface by linking such compounds to the antibody. Thus, the invention also provides methods for localizing *ex vivo* or *in vitro* cells expressing CD20, such as Reed-Sternberg cells (*e.g.*, with a detectable label, such as a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor). Alternatively, the immunoconjugates can be used to kill cells

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which have CD20 expressed on their surface by targeting cytotoxins or radiotoxins to CD20.

The present invention is further illustrated by the following examples which should not be construed as further limiting.

EXAMPLES

B-cell lines used in the examples

Cell line	Origin	Obtained from
Daudi	Negroid Burkitt's Lymphoma	ECACC (85011437)
ARH-77	IgG plasma cell leukemia	DSMZ (ACC 512)
DOHH	Refractory immunoblastic B cell lymphoma	DSMZ (ACC 47)
Raji	Negroid Burkitt's Lymphoma	ECACC (85011429)
SU-DHL-4	B-NHL, diffuse histiocytic lymphoma	DSMZ (ACC 495)
Ramos-EHRB	Burkitt's Lymphoma	ECACC (85030804)
Tanoue	Human B-cell leukemia	DSMZ (ACC 399)

Daudi, ARH-77, DOHH, Raji, Ramos-EHRB, and Tanoue B-cell lines were cultured in RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS) (Optimum C241, Wisent Inc., st. Bruno, Canada), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all Gibco BRL, Life Technologies, Paisley, Scotland).

SU-DHL-4 B-cell line was cultured in the same medium but without sodium pyruvate.

Cultures were maintained at 37 °C in a humidified 5% CO_2 incubator, split and harvested at 80-90% confluence. Medium was refreshed twice a week. At this time cells were split and seeded out to 1-1.5 x 10^6 cells/ml to ensure viability and optimal growth.

Example 1 Production of Human Antibodies Against CD20

HCo7 and KM Mice: Fully human monoclonal antibodies to CD20 were prepared using HCo7 and KM mice which express human antibody genes. In the KM mouse strain, the endogenous mouse kappa light chain gene has been homozygously disrupted as described in Chen *et al.* (1993) *EMBO J.* 12:811-820 and the endogenous mouse heavy chain gene has been homozygously disrupted as described in Example 1 of

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PCT Publication WO 01/09187. This mouse strain carries a human kappa light chain transgene, KCo5, as described in Fishwild *et al.* (1996) *Nature Biotechnology* 14:845-851. This mouse strain also carries a human heavy chain transchromosome composed of chromosome 14 fragment hCF (SC20) as described in WO 02/43478.

The HCo7 mice have a JKD disruption in their endogenous light chain (kappa) genes (as described in Chen *et al.* (1993) *EMBO J.* 12: 821-830), a CMD disruption in their endogenous heavy chain genes (as described in Example 1 of WO 01/14424), a KCo5 human kappa light chain transgene (as described in Fishwild *et al.* (1996) *Nature Biotechnology* 14:845-851), and a HCo7 human heavy chain transgene (as described in US 5,770,429).

HCo7 and KM Mice Immunizations: HCo7 and KM mice were immunized with human CD20 transfected NS/0 cells. For the first immunization, per mouse, $1x10^7$ cells in 150 µl PBS were mixed 1:1 with Complete Freunds Adjuvant and injected intraperitoneally (i.p.). Subsequent i.p. immunizations were done using a similar amount of cells without adjuvant. Three and two days prior to fusion the mice were intravenously boosted with 0.5×10^7 cells suspended in PBS.

The presence of antibodies directed against human CD20 in the serum of the mice was monitored by flow cytometry using FACS analysis, using human CD20 transfected NS/0 cells as well as CD20 negative parental NS/0 cells.

Generation of Hybridomas Producing Human Monoclonal Antibodies to CD20: The mouse splenocytes were isolated from the HCo7 and KM mice and fused with PEG to a mouse myeloma cell line based upon standard protocols. The resulting hybridomas were then screened for human IgG,κ production by ELISA and for CD20 specificity using human CD20 transfected NS/0 and SKBR3 cells by FACS analysis. Single cell suspensions of splenic lymphocytes from immunized mice were fused to one-fourth the number of SP2/0 nonsecreting mouse myeloma cells (ATCC, CRL 1581) with 50% PEG (Sigma). Cells were plated at approximately 1×10^5/well in flat bottom microtiter plate, followed by about two week incubation in selective medium containing 10% fetal bovine serum, 10% P388D1 (ATCC, CRL TIB-63) conditioned medium, 3-5% origen (IGEN) in DMEM (Mediatech, CRL 10013, with high glucose, L-glutamine and sodium pyruvate) plus 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 mg/ml gentamycin and 1x HAT (Sigma, CRL P-7185). After 1-2 weeks, cells were cultured in medium in which the HAT was replaced with HT. Individual wells were then screened by flow cytometry for human anti-CD20 monoclonal IgG antibodies. Once extensive hybridoma growth occurred, medium was monitored usually after 10-14 days. The antibody secreting hybridomas were replated, screened again and, if still positive for

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human IgG, anti-CD20 monoclonal antibodies were subcloned by limiting dilution. The stable subclones were then cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization. One clone from each hybridoma, which retained the reactivity of parent cells (by FACS), was chosen. 5-10 vial cell banks were generated for each clone and stored in liquid nitrogen.

Selection of Human Monoclonal Antibodies Binding to CD20/Primary Screens: To determine the isotype of antibodies, an isotype ELISA was performed. Wells of microtiter plates were coated with 1 μg/ml of mouse anti-human kappa light chain, 50 μl/well in PBS incubated 4 °C overnight. After blocking with 5% chicken serum, the plates were reacted with supernatant and purified isotype control. Plates were then incubated at ambient temperature for 1-2 hours. The wells were then reacted with either human IgG1, IgG2, IgG3 or IgG4-specific Horseradish peroxidase – conjugated probes. Plates were developed and analyzed as described above.

Four hybridoma cell lines were generated, three from fusion of KM mouse and one from fusion of HCo7 mouse, expressing the following antibodies:

2F2: a human monoclonal IgG1,κ antibody with the nucleotide sequences: SEQ ID NOs: 1 and 3 and the amino acid sequences: SEQ ID NOs: 2 and 4.

4C9: a human monoclonal IgG1,κ antibody with exactly the same amino acid sequences as 2F2: SEQ ID NOs: 2 and 4.

7D8: a human monoclonal IgG1,κ antibody with the nucleotide sequences: SEQ ID NOs: 5 and 7 and the amino acid sequences: SEQ ID NOs: 6 and 8.

11B8: a human monoclonal IgG3, κ antibody with the nucleotide sequences: SEQ ID NOs: 9 and 11 and the amino acid sequences: SEQ ID NOs: 10 and 12.

The term "2F2" is used herein to designate both the antibody derived from hybridoma clone 2F2 and the identical antibody derived from hybridoma clone 4C9.

The antibodies of the invention can be switched to other isotypes as determined by the transgenic or transchromosomal non-human animal from which they are derived. In one embodiment of the invention, the 11B8 human monoclonal IgG3,κ antibody can be switched to a human monoclonal IgG1,κ isotype having exactly the same V_H and V_L sequences. In another embodiment, the 2F2 IgG1,κ antibody or 7D8 IgG1,κ antibody can be switched to a human monoclonal IgG2, IgG4, IgA1, IgA2 or IgE isotype having exactly the same V_H and V_L sequences.

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Example 2 Antibody Sequencing of Human Antibodies Against CD20

Sequencing of the V_L and V_H Regions

RNA preparation: Total RNA was prepared from $5x10^6$ cells of all HuMAb CD20 hybridoma cell lines (2F2, 7D8 and 11B8) with RNeasy kit (Qiagen, Westburg, Leusden, Netherlands) according to the manufacturer's protocol.

cDNA preparation of 2F2 and 7D8: 5'-RACE-Ready Complementary DNA (cDNA) of RNA was prepared from 1 µg total RNA, using the SMART RACE cDNA Amplification kit (Clonetech), following the manufacturer's protocol.

V_H and V_L regions were amplified using an advantage HF 2 PCR Kit (Clonetech, BD) and using the following primers:

V_K RACE2	5' GCA GGC ACA CAA CAG AGG CAG TTC CAG ATT TC	anneals in C-kappa
V_H RACE2	5' GCT GTG CCC CCA GAG GTG CTC TTG GAG G	anneals in C_{H1}

cDNA preparation of 11B8: Complementary DNA (cDNA) of RNA from 11B8 cells was prepared from 3 µg total RNA with AMV Reverse Transcriptase with buffer (Roche Diagnostics GmbH, Mannheim, Germany), oligo $d(T)_{15}$ (Promega, Madison, WI, USA), dNTP (Roche Diagnostics GmbH, Mannheim, Germany) and RNAsin (Promega) according to the manufacturer's protocol (2000, version 3).

PCR primers used to amplify V_H and V_L regions for cloning:

Primer pairs used:

V_H: FR1 5' primers

AB62	CAg gTK CAg CTg gTg CAg TC
AB63	SAg gTg CAg CTg KTg gAg TC
AB65	gAg gTg CAg CTg gTg CAg TC

V_H leader 5' primers

AB85	ATg gAC Tgg ACC Tgg AgC ATC
AB86	ATg gAA TTg ggg CTg AgC Tg
AB87	ATg gAg TTT ggR CTg AgC Tg
AB88	ATg AAA CAC CTg Tgg TTC TTC
AB89	ATg ggg TCA ACC gCC ATC CT

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V_H 3' primer

AB90	TgC CAg ggg gAA gAC CgA Tgg

V_K:

FR1 5' primers

AB8	RAC ATC CAg ATg AYC CAg TC
AB9	gYC ATC YRg ATg ACC CAg TC
AB10	gAT ATT gTg ATg ACC CAg AC
AB11	gAA ATT gTg TTg ACR CAg TC
AB12	gAA ATW gTR ATg ACA CAg TC
AB13	gAT gTT gTg ATg ACA CAG TC
AB14	gAA ATT gTg CTg ACT CAg TC

V_K leader 5' primers

AB123	CCC gCT Cag CTC CTg ggg CTC CTg
AB124	CCC TgC TCA gCT CCT ggg gCT gC
AB125	CCC AgC gCA gCT TCT CTT CCT CCT gC
AB126	ATg gAA CCA Tgg AAg CCC CAg CAC AgC

V_K 3' primer

AB16	Cgg gAA gAT gAA gAC AgA Tg

wherein K = T or G, S = C or G, R = A or G, Y = C or T, and W = A or T.

PCR conditions used to amplify V_H and V_L regions for cloning 2F2 and 7D8: Polymerase chain reactions (PCR) were performed with HF polymerase mix (Clonetech.) on a T1 cycler (Biometra, Westburg).

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PCR conditions:

94 °C 30 sec 5 cycles
72 °C 1 min

94 °C 30 sec
70 °C 30 sec 5 cycles
72 °C 1 min

94 °C 30 sec
68 °C 30 sec 27-30 cycles
72 °C 1 min

PCR conditions used to amplify V_H and V_L regions for cloning 11B8: Polymerase chain reactions (PCR) were performed with AmpliTaq polymerase (Perkin Elmer) on a T1 Cycler (Biometra, Westburg, Leusden, Netherlands).

PCR cycling protocol:

94 °C 2 min
11 cycles 94 °C 30 sec
65 °C 30 sec, minus 1 °C per cycle
72 °C 30 sec
30 cycles 94 °C 30 sec
55 °C 30 sec
72 °C 30 sec
72 °C 10 min
cool down to 4 °C

Cloning of V_H and V_L in pGEMT-Vector System II (2F2, 7D8, and 11B8): After analysing the PCR products on an agarose gel, the products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Westburg, Leusden, Netherlands). Two independently amplified PCR products of each V_H and V_L region were cloned in pGEMT-Vector System II (Promega) according to manufacturer's protocol (1999, version 6).

After transformation to *E. coli* JM109, individual colonies were screened by colony PCR using T7 and SP6 primers, 30 annealing cycles at 55 °C. Plasmid DNA from colonies was purified using Qiaprep Spin miniprep kit (Qiagen). To further

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analyse the V_H and V_L regions, a *Nco*1/*Not*1 (NE Biolabs, Westburg, Leusden, Netherlands) digestion was performed and analysed on agarose gel.

Sequencing (2F2, 7D8 and 11B8): The V-regions regions were sequenced after cloning in the pGEMT-Vector System II. Sequencing was performed at Baseclear (Leiden, Netherlands). The sequences were analyzed by aligning germline V-gene sequences in Vbase (www.mrc-cpe.cam.ac.uk/imt-doc/public/intro.htm).http://www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901.

The sequences obtained are shown in Figures 53-58.

Example 3 Recombinant production of 2F2 and 11B8 in GS-NS/0 cell line

2F2T: The heavy chain and light chain variable regions of the 2F2 antibody were amplified, using PCR, from a standard cloning vector, pGem-5Zf (Promega), using primers which included an optimal Kozak sequence and suitable restriction sites to clone the fragments in the GS constant region vectors pCONγ1f and PCONκ (Lonza).

After amplification, the fragments were purified and digested with the restriction enzymes for cloning and ligated in the two vectors. The heavy chain variable fragment was digested with *Hind* III and *Bsi* WI and ligated into the pCONγ1f vector which had been digested with *Hind* III and *Bsi* WI, and dephosphorylated with alkaline phosphatase. The light chain variable fragment was digested with *Hind* III and *Apa* I and ligated into the PCONκ vector which had been digested with *Hind* III and *Apa* I, and dephosphorylated with alkaline phosphatase. The pCONγ1f/variable-heavy and PCONκ/variable-light vectors are shown in Figures 1 and 2, respectively. Transformed *E. coli* colonies were checked by colony PCR and 2 positive colonies of each the heavy chain (HC) and light chain (LC) construct were grown for plasmid isolation. Isolated plasmid of these 4 clones was sequenced to confirm the sequence. Both of the HC clones and one of the LC clones were found to have the correct sequences.

The two HC and one LC constructs were combined to give two combinations of LC-HC and transiently co-transfected in CHO-K1 cells to check the constructs for proper production of 2F2 antibody. Normal production levels were reached for all combinations in this expression experiment and 1 clone of each of the HC and LC constructs were chosen for construction of a double-gene vector.

Standard cloning procedures were used to combine the HC and LC constructs in a double-gene cloning vector, designated pCONγ1f/κ2F2, by ligating the complete expression cassette from the heavy chain vector, pCONγ1f/variable-heavy,

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into the light chain vector, pCONκ/variable-light. The pCONγ1f/κ2F2 vector is shown in Figure 3.

This construct was again functionally tested in a transient transfection in CHO-K1 cells and showed normal expression levels.

The variable regions of the pCONγ1f/κ2F2 plasmid were sequenced to reconfirm the correct sequences.

Linear plasmid was prepared for stable transfections by digesting pCONγ1f/κ2F2 with a unique restriction enzyme, *Pvu I,* cutting outside regions vital for expression. Complete linearization was confirmed by agarose gel electrophoresis and the DNA was purified and stored at -20 °C until use.

Six transfections of NS/0 host cells were performed, by electroporation with plasmid DNA, using the above linear DNA plasmid. Following transfection, the cells were distributed into 96-wells plates and incubated. Selective medium (containing 10% dialysed fetal calf serum (dFCS) and 10 μM of the GS-inhibitor L-methionine sulphoximine but lacking glutamine) was added and the plates were monitored to determine when the non-transfected cells died to leave foci of transfected cells. For further details concerning GS vector systems, see WO 87/04462. The transfected plates were incubated for approximately three weeks to allow colony formation. The resulting colonies were examined microscopically to verify that the colonies were of a suitable size for assay (covering greater than 60% of the bottom of the well), and that only one colony was present in each well. Cell supernatants from 436 transfectants were screened for assembled antibody by IgG,κ-ELISA. Using this data, 111 transfectants were selected for progression and further assessment in static culture. Cultures of the selected cell lines were expanded and adapted to low-serum containing medium (containing bovine serum albumin (BSA) and added 1% dFCS) and a further assessment of productivity in static culture was undertaken (ELISA and measurement of percentage confluence). The 65 highest ranking cell lines were selected for progression. A preliminary assessment of the productivity of the selected cell lines was made in batch shake flask suspension culture in low serum-containing medium (containing BSA and added 1% dFCS). Based upon harvest antibody concentration (by ELISA) and acceptable growth characteristics, 30 cell lines were selected for further evaluation in serum-free medium using a batch shake flask suspension culture. The 10 cell lines that produced the highest antibody concentrations were further evaluated in duplicate fed-batch shake flask suspension cultures in serum-free medium. Product concentrations at harvest were determined by protein A high performance liquid chromatography (HPLC), according to well-known standard methods. All cell lines produced 2F2 antibody (denoted 2F2T) in good yields in the range of from 671-1333 mg/L as determined by protein A HPLC.

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11B8T: In a similar way a GS-NS/0 cell line was established for recombinant production of 11B8 (denoted 11B8T) modifying the transfection procedure slightly as follows.

Four transfections of NS/0 host cells were performed, by electroporation with plasmid DNA, using the above linear DNA plasmid. After examining the resulting colonies microscopically to verify that the colonies were of a suitable size for assay (covering greater than 60% of the bottom of the well) and that only one colony was present in each well, cell supernatants from 596 transfectants were screened for assembled antibody by IgG,κ-ELISA. Using this data, 100 transfectants were selected for progression and further assessment in static culture. Cultures of the selected cell lines were expanded and adapted to low-serum containing medium (containing bovine serum albumin (BSA) and added 1% dFCS) and a further assessment of productivity in static culture was undertaken (ELISA and measurement of percentage confluence). The 60 highest ranking cell lines were selected for progression, and an additional 13 cell lines for which productivity data was unavailable were also progressed. A preliminary assessment of the productivity of the selected cell lines was made in batch shake flask suspension culture in low serum-containing medium (containing BSA and added 1% dFCS). Based upon harvest antibody concentration (by ELISA) and acceptable growth characteristics, 10 cell lines were selected for further evaluation in duplicate fed-batch shake flask suspension cultures in low serum-containing medium (containing BSA and added 1% dFCS). Product concentrations at harvest were determined by protein A high performance liquid chromatography (HPLC), according to well-known standard methods. Based on this one of the cell lines was discarded. The resulting 9 cell lines all produced 11B8 antibody (denoted "11B8T") in good yields in the range of from 354-771 mg/L as determined by protein A HPLC.

Example 4 Comparison of hybridoma-derived 2F2 and transfectoma-derived recombinant 2F2T

By use of gel electrophoresis (SDS-PAGE and native agarose gel electrophoresis) it was shown that 2F2 and 2F2T are of the same size, and only slightly differ in electric charge.

Furthermore, 2F2 and 2F2T bind to CD20-transfected NS/0 cells and Raji cells with similar affinity as measured by flow cytometry using FACScalibur™ (Becton Dickinson, San Diego, CA, USA). No binding to non-transfected NS/0 cells was observed demonstrating the specificity of 2F2 and 2F2T. 2F2 and 2F2T also induce CDC in a concentration-dependent manner to the same extent in ARH-77 cells (IgG plasma cell leukemia), Daudi cells, DOHH cells (refractory immunoblastic B cell

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lymphoma progressed from follicular centroblastic/centrolytic lymphoma, DSMZ, Braunschweig, Germany) and Raji cells, measuring cell lysis (number of PI-positive cells) by flow cytometry (FACScalibur). In a second experiment, the concentrations of 2F2 and 2F2T were kept constant while serum was added in different concentrations. No significant differences between 2F2 and 2F2T were observed.

Finally, 2F2 and 2F2T bound to cell-associated CD20 binds complement factor C1q strongly and to the same extent. The experiment was performed in Daudi cells, DOHH cells, and Raji cells using fluorescein-conjugated anti-C1q polyclonal antibodies for detecting binding of C1q.

Example 5 Binding Characteristics of Human Antibodies Against CD20

Binding to different cell lines: NS/0, NS/0 transfected with human CD20, Daudi and Raji cells were incubated for 30 min at 4 °C with culture supernatant containing human antibodies 2F2, 7D8, and 11B8 followed by incubation with FITC-conjugated anti-human IgG Ab. Binding was assessed by flow cytometry using a FACScalibur flow cytometer. Fluorescence intensities were compared with negative control isotype matched samples. As shown in Figure 4, all three antibodies bound to NS/0 cells transfected with human CD20, whereas no binding was observed to parental, non-transfected NS/0 cells. All three antibodies also bound to the two different Burkitt lymphoma B cell lines (Raji and Daudi) indicating that 2F2, 7D8, and 11B8 are CD20 specific. Supernatant containing 7D8 or 11B8 were tested under non-saturating conditions, therefore, lower mean fluorescence intensities compared to 2F2 are observed.

EC_{50} value of 2F2 as determined by flow cytometry: In order to determine the apparent affinity of 2F2 for CD20 expressed on human B cells, a binding curve was made of 2F2 using isolated PBMCs from three human donors and gating of CD3-negative cells. The isolated PBMCs were incubated for 1 hour with a concentration range of FITC labeled 2F2 and analysed on FACS, and the mean fluorescence intensity (MFI) determined. The MFI values are shown in Figures 5A and 5B as a function of the antibody concentration. The EC_{50} values were calculated by use of Graph Pad Prism 3.02 by non-linear regression. The EC_{50} value of 2F2 in humans was similar for all three donors with a mean (± s.e.m.) of 287 ± 12.7 ng/ml (1.9 ± 0.1 nM).

Binding of ^{125}I-labeled mAbs to CD20 expressing cells: mAbs were iodinated using Iodobeads (Pierce Chemical Co., Rockford, IL). ^{125}I-labelled mAbs were serially diluted and incubated with Ramos-EHRB (cells for 2 hours at 37 °C in the

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presence of sodium azide and 2-deoxyglucose to prevent endocytosis. The cell bound and free ^{125}I labeled mAbs were then separated by centrifugation at 14,000 x g for 2 min through a mixture of phthalate oils, allowing rapid separation without disturbing the binding equilibrium. The pelleted cells together with bound antibody were then counted using a gamma counter (Wallac UK Ltd, Milton Keynes, UK).

As shown in Figure 6, 2F2 and 11B8 exhibit similar K_D (or similar saturation points) indicating that both antibodies bind with similar affinity. However, 11B8 saturates at a lower level than 2F2 indicating that it recognized a different form of CD20. This is also in agreement with a further experiment showing that a similar number of 2F2 and rituximab antibody molecules binds to CD20 on Ramos-EHRB cells and Daudi cells, as shown by the similar levels of binding saturation (approximately 2-3 x 10^5 antibody molecules per cell). 11B8 and B1, in contrast, saturate at half this level and only about 1-2 x 10^5 antibody molecules bind to the Ramos-EHRB cells (Figure 7A) and Daudi cells (Figure 7B).

To exclude the possibility that the iodinated antibodies bind via Fc-receptors, binding curves were confirmed by use of anti-CD20 $F(ab')_2$ fragments. Again, similar numbers of 2F2 and rituximab-$F(ab')_2$ fragments bound to both Ramos-EHRB and Daudi cells. Also in these experiments, the number of 2F2 or rituximab antibody molecules bound to Ramos-EHRB cells and Daudi cells saturates at approximately twice the number of 11B8 and B1 molecules bound to the cells.

Dissociation rate: To determine the dissociation rate of the mAbs, Ramos-EHRB cells (final volume of 1 ml in the presence of azide/2DOG) were incubated for 2 hours at 37 °C with 2 μg/ml ^{125}I mAbs to achieve maximum binding. Following centrifugation in a microfuge (2000 rpm for 2 min), the supernatant was removed, the pellet quickly resuspended in 1 ml medium, and immediately transferred to 9 ml medium at 37 °C in a 15 ml conical tube. At various times over the next 2 hours, 0.4 ml samples were removed and separated on phthalate oils to determine the level of radiolabeled mAbs remaining on the cell surface. As shown in Figure 8, both 2F2 and 11B8 dissociated significantly more slowly from CD20 than rituximab or B1.

Dissociation rates of anti-CD20 $F(ab)_2$ fragments: Ramos-EHBR cells were saturated with 2 μg/ml of ^{125}I-labeled $F(ab)_2$ fragments of 2F2, 11B8, and rituximab, respectively. The Ramos-EHBR cells were washed and incubated in the presence of a high concentration of the unlabeled antibody. The maximal (initial) binding to Ramos-EHRB cells was set at 100%. At several time points over the next 3 hours following loading, 0.4 ml samples were removed and separated on phthalate oil to determine the levels of radiolabeled mAb remaining on the cell surface. As can be seen

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from Figure 9, 2F2 and 11B8 dissociated much more slowly from the surface of CD20 than rituximab. At 90 min, approximately 50% of the $F(ab)_2$ rituximab molecules were bound to the cell, whereas half of the $F(ab)_2$ 2F2 molecules were dissociated after 3 hours. The k_d (k_{off}) values for 2F2, 11B8T, and rituximab are calculated as follows:

$F(ab)_2$ 2F2: $k_d = \ln 2/t_{½}$ (sec) = ln 2/10800 (sec) = 6.4×10^{-5} sec^{-1}

$F(ab)_2$ 11B8T: $k_d = \ln 2/t_{½}$ (sec) = ln 2/9000 (sec) = 7.7×10^{-5} sec^{-1}

$F(ab)_2$ rituximab: $k_d = \ln 2/t_{½}$ (sec) = ln 2/5400 (sec) = 1.3×10^{-4} sec^{-1}

Anti-CD20 mAb functional off rates: The impact of the slow 2F2 dissociation rate compared to rituximab was assessed in a functional CDC assay. To this end, Daudi or SU-DHL4 cells were pre-incubated with 10 μg/ml anti-CD20 mAb or an isotype control antibody, washed and incubated in medium for different time points. At these time points after start of the assay, samples were incubated with complement (normal human serum 20 vol/vol%) and then incubated for another 45 min at 37 °C. Thereafter, cell lysis was determined on FACS by using PI (propidium iodide) staining method. The % lysed cells (PI-positive cells) are shown in Figure 10A (Daudi cells) or Figure 10B (SU-DHL4 cells) as a function of incubation time. 2F2 induced high CDC in both cell lines, and still lysed up to 90% of the cells after 6 hours, indicating that the CD20 saturation of the cells remained sufficiently high to induce complement-mediated lysis of most of the cells. Rituximab, in contrast and in agreement with the above dissociation rate studies, dissociated rapidly from the cells and failed to induce specific lysis following the 6 hour incubation period. 11B8 was used as a control and did not induce CDC.

Example 6 CDC of Human Antibodies Against CD20

Serum preparation: Serum for complement lysis was prepared by drawing blood from healthy volunteers into autosep gel and clot activator vacutainer tubes (BD biosciences, Rutherford, NJ) which were held at room temperature for 30-60 min and then centrifuged at 3000 rpm for 5 min. Serum was harvested and stored at -80 °C.

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Flow cytometry: For flow cytometry a FACScalibur flow cytometer was used with CellQuest pro software (BD Biosciences, Mountain view, CA). At least 5000 events were collected for analysis with cell debris excluded by adjustment of the forward sideward scatter (FCS) threshold.

CDC kinetics: In a first set of experiments (n=3) the kinetics of CDC of five different B-cell lines, *i.e.*, Daudi, SU-DHL-4, Raji, DOHH and ARH-77, were determined by adding 10 μg/ml 2F2, rituximab and an IgG control antibody, respectively, for 10 min before human serum was added. At several time intervals (up to one hour) after induction of CDC, the cells were suspended in PI solution and cell lysis (number of PI-positive cells) was measured by flow cytometry. The results are depicted in Figures 11A (ARH-77 cells), 11 B (Daudi cells), 11C (Raji cells), 11D (DOHH) and 11 E (SU-DHL-4). As seen, addition of antibodies induced cell lysis within 5 min. Interestingly, addition of 2F2 resulted in a marked cell lysis of more than 80% in all five B-cell lines. Rituximab induced more than 80% cell lysis only in the SU-DHL-4 and Daudi cell lines, whereas the cell lysis of the DOHH cell line was ~50%, and less than 20% in the ARH-77 and Raji cell lines. No lysis was observed with the IgG control antibody (data only shown in Figure 11B).

CDC serum titration: In a separate set of experiments (n=5), NHS (normal human serum) was titrated at two different antibody concentrations of 0.5 μg/ml and 5 μg/ml. Cells were pre-incubated with 2F2 or rituximab for 10 min, before a concentration range of NHS was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 12A-D show the percentage of lysed (PI-positive) cells as a function of NHS concentration. Figure 12A shows cell lysis of Daudi cells, Figure 12B cell lysis of ARH-77 cells, Figure 12C cell lysis of DOHH cells, and Figure 12D cell lysis of Raji cells. Increased lysis of cells was observed with increased NHS concentration. Addition of 2F2 caused maximal lysis of Daudi cells at the highest NHS and antibody concentration. Rituximab induced about 50% cell lysis of Daudi cells at the highest NHS concentration.

In ARH-77 cells, only the highest concentration of NHS and 2F2 led to approximately 75% cell lysis. Lower antibody concentrations were insufficient to induce ARH-77 cell lysis. Rituximab was not able to induce cell lysis of ARH-77 cells in this experiment.

2F2 was able to induce NHS-concentration dependent cell lysis of DOHH cells at both the high and the low concentration, whereas rituximab was not able to induce lysis under these conditions.

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Finally, 2F2 induced NHS-concentration-dependent lysis of Raji cells, which was only apparent by use of 5 μg/ml mAb. No lysis was observed with rituximab.

In these experiments, no lysis was observed with the isotype control antibody (data not shown).

CDC antibody titration: To measure the ability of the anti-CD20 antibodies to induce CDC at low concentrations, an experiment was performed where the antibodies were titrated (n=6). Various cell lines were pre-incubated with a concentration range of 2F2 and rituximab, respectively, for 10 min before NHS was added. After 45 min incubation at 37 °C (when maximal lysis occurs) the cells were resuspended in PI solution and cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 13A (Daudi cells), 13B (DOHH cells), 13C (ARH-77 cells), and 13D (Raji cells) show the percentage of lysed (PI-positive) cells as a function of antibody concentration. Both 2F2 and rituximab induced a concentration-dependent increase in cell lysis. 2F2 induced more than 80% lysis of Daudi cells upon addition of 2 μg/ml, whereas with rituximab this level was not reached even after addition of 10 μg/ml. Furthermore, 2F2 induced more than 80% lysis of DOHH cells at 0.4 μg/ml, whereas minimal lysis was observed with rituximab at this concentration. The maximal lysis of DOHH cells with rituximab (~30% of total cell analyzed) was reached at 10 μg/ml. Induction of lysis of ARH-77 and Raji cells by 2F2 was lower, but still ~70% lysis was reached at an antibody concentration of 10 μg/ml. At its highest concentration, rituximab induced lysis in only ~23% of ARH-77 cells, and in only ~6% of Raji cells.

In a similar experiment, 2F2, 2F2T, 11B8T, and rituximab were investigated for their ability to induce CDC of Daudi and Raji cell lines, see Figures 14A and 14B. Also in this experiment more than 80% lysis of Daudi cells was observed with (transfectoma-derived) 2F2T at 10 μg/ml, whereas rituximab reached only to 60% lysis even at 10 μg/ml, cf. Figure 14A. Lysis of Daudi cells with 2F2T was identical to the lysis obtained with hybridoma-derived 2F2.

Lysis of Raji cells was more difficult, but again both 2F2 and 2F2T induced lysis of Raji cells to a similar extent (Figure 14B). Rituximab was not able to induce CDC of Raji cells which is in agreement with the experiment shown in Figure 13D.

As can be seen from Figures 14A and 14B neither Daudi nor Raji cells were susceptible to CDC by 11B8T. B1 induced lysis of Daudi cells, but only to a small extent, and was not able to induce lysis of Raji cells.

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CDC activity of anti-CD20 in Daudi cells: To determine the CDC activity of each antibody, elevated membrane permeability was assessed using FACS analysis of propidium iodide (PI)-stained cells. Briefly, the Daudi cells were washed and resuspended in RPMI/1% BSA at 1 x 10^6 cells/ml. Various concentrations of human monoclonal antibodies were added to the Daudi cells and allowed to bind to CD20 on the cells for 10-15 min at room temperature. Thereafter, serum as a source of complement was added to a final concentration of 20% (v/v) and the mixtures were incubated for 45 min at 37 °C. The cells were then kept at 4 °C until analysis. Each sample (150 µl) was then added to 10 µl of PI solution (10 µg/ml in PBS) in a FACS tube. The mixture was assessed immediately by flow cytometry. As shown in Figure 15A, 2F2 and 7D8 showed superior CDC activity compared to rituximab.

In a second experiment, cells were labeled with human monoclonal antibodies as above, then washed and incubated in PBS for 45 min at 37 °C prior to the addition of human serum. This ensured that only antibody bound to the cell at the time of serum addition was available to activate complement for cell lysis. As shown in Figure 15B, decreased CDC activity was found for rituximab compared to 2F2 and 7D8 indicating that the human antibodies (2F2 and 7D8) are not affected by washing the cells prior to the addition of serum.

CDC activity of anti-CD20 in Raji cells: CDC activity was assessed using Raji cells which have relatively high surface expression of CD55 and CD59 and, therefore, are more resistant to complement attack. Human antibodies were added to Raji cells and allowed to bind for 15 min. Human serum (20%) was added and the mixtures incubated for 45 min at 37 °C. As shown in Figure 16A, rituximab was ineffective in mediating CDC of Raji cells whereas significant levels of cell lysis occurred in Raji cells opsonized with 2F2 or 7D8. Accordingly, 2F2 and 7D8 have a unique capacity to lyse CD55/59 positive target cells.

In a separate experiment, Raji cells were pre-incubated with saturating concentrations of anti-CD55 mAb (final concentration of 5 µg/ml) and anti-CD59 mAb (final concentration of 5 µg/ml) to block the effects of these complement defense molecules. Human anti-CD20 antibodies were then added along with serum (20%) as above for 45 min at 37 °C. As shown in Figure 16B, the blockade of CD55 and CD59 molecules resulted in almost 100% lysis of Raji cells with human antibodies 2F2 or 7D8 whereas only a 25% increase in cell lysis was observed using rituximab.

Role of complement inhibitors I – Expression of surface molecules: Since complement inhibitors such as CD55 and CD59 appear to play an important role in susceptibility to rituximab-induced CDC, an experiment was performed to determine

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the expression of these molecules on the B-cell lines under investigation (Raji, Daudi, DOHH, ARH-77, and SU-DHL-4).

The cells were stained with FITC-conjugated anti-CD55, anti-CD59 and anti-CD20 antibodies and molecules expression was analyzed by flow cytometry. The results are shown in the below Table 1.

Table 1

Expression	CD20	CD55	CD59
ARH-77	++	++++	++
Raji	+	++	+++
DOHH	++	+++	++
SU-DHL-4	+++	+	++
Daudi	++	+	+

Role of complement inhibitors II – Blockade of CD55 and CD59: To further study the roles of CD55 and CD59 in anti-CD20-induced CDC, both complement inhibitor molecules were blocked by specific antibodies prior to induction of CDC (n=3). Raji cells were used because only partial lysis was induced by 2F2 alone. Raji cells (1 x 10^5 cells/50 µl) were pre-incubated with a concentration range of 2F2 and rituximab together with anti-CD55 (5 µg/ml) or anti-CD59 (5 µg/ml) antibodies for 10 min, before pooled NHS (20%) was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. Figures 17A-C show the percentage of lysed (PI-positive) cells as a function of antibody concentration, and show one experiment which is exemplary of three experiments. Figure 17A shows incubation of Raji cells with anti-CD55 antibody, Figure 17B incubation of Raji cells with anti-CD59 antibody, and Figure 17C incubation of Raji cells with anti-CD55 and anti-CD59 antibodies.

As can be seen in Figure 17A, addition of anti-CD55 antibody did not influence 2F2 or rituximab-induced CDC. Addition of anti-CD59 antibody increased susceptibility of the cells to both 2F2 and to rituximab with ~30% (Figure 17B). Addition of both anti-CD55 and anti-CD59 further enhanced anti-CD20-induced lysis of cells with ~30% (Figure 17C).

Role of complement factors, as determined by flow cytometry I – C1q binding: Anti-CD20 antibodies (2F2 and rituximab) and an isotype control antibody were added to various B-cell lines. After 10 min incubation, NHS (1 vol/vol%) was added. After further incubation for 10 min at 37 °C and washing of the cells, the supernatant was discarded and the cell pellet was incubated with FITC-conjugated anti-

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C1q antibody. Data show mean fluorescence intensity of cells stained with C1q and are depicted in Figures 18A (Daudi), 18B (ARH-77), 18C (DOHH), and 18D (Raji) (n=6). The results indicated antibody concentration-dependent increase in binding of C1q by 2F2, irrespective of the B-cell line investigated. Moreover, C1q binding by 2F2 was always higher than binding by rituximab, in all cell lines tested. No increase in mean fluorescence was observed with the isotype control antibody (data not shown).

Role of complement factors, as determined by flow cytometry II – Complement activation via the classical route: Fixation of C4c to antibody-coated cells is an indication of activation of complement activation via the classical route. Anti-CD20 antibodies (2F2 and rituximab) and an isotype control antibody were added to various B-cell lines. After 10 min incubation at 37 °C, NHS (1 vol/vol%) was added. After further incubation and washing of the cells, the supernatant was discarded and the cell pellet was incubated with FITC-conjugated anti-C4c antibody. Data show mean fluorescence intensity of cells stained with C4c and are depicted in Figures 19A (Daudi), 19B (ARH-77), 19C (DOHH), and 19D (Raji) (n=6). Complement factor C4c fixation to 2F2 was demonstrated in all B-cell lines tested (n=3), with a maximum reached at ~1 µg/ml of antibody. Fixation of C4c after 2F2 binding was much higher than after rituximab, irrespective of the cell line tested. No increase in mean fluorescence was observed with the isotype control antibody (data not shown).

CDC in heat-inactivated serum: Cells (Daudi cells, ARH-77 cells or Raji cells) and antibodies (rituximab, 2F2, 2F2T, 11B8, and isotype control antibody HuMab-KLH IgG1) were pre-incubated in a concentration range of anti-CD20 antibodies for 10 min, before NHS (active or heat-inactivated in a water bath at 57 °C at 30 min) was added. At 45 min after induction of CDC, cells were resuspended in PI solution. Cell lysis (number of PI-positive cells) was measured by flow cytometry. No lysis of the cells was observed in the presence of heat-inactivated serum, irrespective of the cell-line and CD20-antibody used, no CDC was observed in the presence of heat-inactivated serum.

Example 7 ADCC of Human Antibodies Against CD20 ADCC Assay I

Enrichment of human neutrophils: Polymorphonuclear cells (neutrophils, PMNs) were enriched from heparinized whole blood. Blood was diluted twice in RPMI 1640 and was layered on Ficoll (Lymphocyte Separation Medium 1077 g/ml, 710 g, RT, 20 min; BioWhittaker, cat. 17-829E, lot no. 0148 32) and centrifuged at 2000 rpm for 20 min. The mononuclear cell layer was removed, and erythrocytes within the pellet

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containing neutrophils were hypotonically lysed using ice-cold NH_4Cl solution (155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). The remaining neutrophils were washed twice and resuspended in RPMI 1640 supplemented with 10% FCS (RPMI-10).

Enrichment of human peripheral blood mononuclear cells: Human blood was diluted twice in RPMI 1640 and blood cells were layered on Ficoll (Lymphocyte Separation Medium 1077 g/ml, 710 g, RT, 20 min; BioWhittaker, Cambrex Bio Science Verviers, Verviers, Belgium, cat. 17-829E, lot no. 0148 32). Peripheral blood mononuclear cells (MNCs) were collected from the interphase, washed and resuspended in RPMI 1640 culture medium supplemented with 10% FCS, 2 mM L-glutamine, 5 U/ml penicillin, 50 µg/ml streptomycin (all derived from BioWhittaker) to which 25 mM HEPES (BioWhittaker) was added.

ADCC set up: Target B-cells (freshly isolated B-cells or from B-cell lines) were labeled with 20 µCi ^{51}Cr (Amersham Biosciences, Uppsala, Sweden) for 2 hours. After extensive washing in RPMI-10, the cells were adjusted to 1 x 10^5 cells/ml. Whole blood or isolated effector cells (50µl; MNCs, PMNs) or plasma (50 µl), sensitizing antibodies (50 µl), and RPMI-10 (50 µl) were added to round-bottom microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Assays were started by adding target cells (50 µl) giving a final volume of 200 µl. For isolated effector cells, an effector to target (E:T) ratio of 40:1 was used. For whole blood, an amount of 33 vol/vol% was used corresponding to an estimated effector to target ratio of 40:1. After incubation (3 hours, 37 °C), assays were stopped by centrifugation, and ^{51}Cr release from triplicates was measured in counts per minute (cpm) in a scintillation counter. Percentage of cellular cytotoxicity was calculated using the following formula:

% specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) x 100

with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release measured in the absence of sensitizing antibodies and effector cells.

Statistics: Data were analyzed by one-way ANOVA, followed by Tukey's multi comparison post-hoc test. Analysis was performed using Graph Pad Prism (version 3.02 for Windows, Graph Pad Software, San Diego, CA, USA).

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Lysis of ARH-77 cells: In a first set of experiments, ARH-77 cells were used as target cells (Figure 20). Addition of 2F2 (n=3), rituximab (n=3) or 11B8T (n=1) resulted in MNC-mediated lysis of ARH-77 cells of approximately 50%. No specific lysis was observed in the presence of neutrophils. Addition of plasma (to evaluate the role of complement) induced lysis of ARH-77 cells after incubation with 2F2, but not after incubation with rituximab ($p<0.05$, 2F2 vs. no antibody, ANOVA) or 11B8T. In the presence of whole blood, lysis of ARH-77 cells increased after incubation with 2F2 ($p<0.05$, 2F2 vs. rituximab and 2F2 vs. no antibody, ANOVA), but not with rituximab. Specific lysis induced by rituximab was in fact very low in the presence of whole blood. 11B8T induced cell lysis of approximately 25% (n=1) in the presence of whole blood. In the absence of antibody, non-specific lysis of 10-15% was observed.

Lysis of B-CLL cells: In a second set of experiments, chronic B-lymphocytic leukaemia (B-CLL) cells obtained from B-CLL patients (n=12) were subcloned for 5 rounds and then used as target cells in the experiment (Figure 21). In the absence of antibody, no specific lysis was observed, but addition of 2F2, 11B8T or rituximab (10 μg/ml) increased MNC-mediated specific lysis to 10-20% ($p<0.001$, ANOVA). Incubation of target cells with plasma and 2F2 induced specific lysis of B-CLL cells, whereas no specific lysis was observed with 11B8T or rituximab ($p<0.001$, ANOVA). Moreover, 2F2 mediated specific lysis of B-CLL cells after incubation in whole blood. No specific lysis of B-CLL cells by whole blood was observed with 11B8T ($p<0.01$, ANOVA) or rituximab ($p<0.001$, ANOVA). No specific lysis was observed in the presence of neutrophils.

Because rituximab was able to mediate effective ADCC but not CDC of the tumor cells tested, it is likely that whole blood-induced B-cell lysis by 2F2 is mediated via complement.

Lysis of hairy cell leukaemia (HCL) cells: In a third set of experiments lysis of HCL cells by 2F2, 11B8T, and rituximab by ADCC or in the presence of plasma or whole blood was determined. Data are shown in Figure 22. Whereas neutrophils could not mediate ADCC irrespective of the mAb used, 11B8T was able to induce MNC-mediated lysis of HCL cells more efficiently than 2F2 ($p<0.001$, ANOVA) or rituximab ($p<0.05$, ANOVA). 2F2 and rituximab were not able to induce MNC-mediated lysis of HCL cells. Plasma-mediated lysis of the cells was strongly enhanced with 2F2, as compared to rituximab ($p<0.05$, ANOVA), 11B8T ($p<0.01$, ANOVA) or without antibody ($p<0.001$, ANOVA). When lysis induced by anti-CD20 in the presence of whole blood was studied, 2F2 induced complete lysis of cells, and was

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superior to rituximab ($p<0.01$, ANOVA), 11B8T or no antibody added ($p<0.001$, ANOVA).

Lysis of B-ALL cells: Using cells from two patients the ability of 2F2 and rituximab to induce lysis B-ALL cells by ADCC or complement was investigated (Figure 23). As was observed in the previous experiments, 2F2 and rituximab induced MNC-mediated ADCC of B-ALL cells to a similar extent. But again 2F2 was able to induce plasma- and whole blood-mediated lysis of B-ALL cells, whereas rituximab was not.

Lysis of follicular lymphoma cells: When lysis of follicular lymphoma cells (n=2) was investigated, a different picture emerged (Figure 24). A minor PMN-mediated lysis of cells with 2F2 was observed, and both 2F2 and rituximab were not able to induce MNC-mediated ADCC. 11B8T was still able to induce MNC-mediated lysis of approximately 20%. Although a relatively high plasma-mediated lysis was induced by rituximab, complete plasma-mediated lysis was observed with 2F2. Also with whole blood, complete lysis was observed with 2F2, whereas 70% lysis with rituximab. Minimal plasma- or whole blood-mediated lysis by 11B8T was observed.

Lysis of primary mantle cell lymphoma cells: Specific lysis of mantle cell lymphoma cells was more difficult to induce (n=1, Figure 25). Minimal or no lysis by 2F2, 11B8T or rituximab was observed after addition of PMN or MNC and CD20 mAbs. However, 2F2 was still able to induce approximately 40% lysis by plasma or whole blood, whereas with rituximab only 10-20% of the cells were lysed. 11B8T was not able to induce lysis of primary mantle cell lymphoma cells.

Antibody concentration-dependent lysis of ARH-77 cells in whole blood: In a further experiment (n=4) dose-dependency regarding the induction of ADCC on ARH-77 cells in the presence of whole blood was analyzed. As can be seen in Figure 26, titration of 2F2 induced a dose-dependent increase in the percentage of specific lysis ($p<0.05$: treatment-effect, two-way ANOVA) of ARH-77 cells. No specific lysis of ARH-77 cells was observed with rituximab.

ADCC assay II

Preparation ^{51}Cr-labeled target cells: ARH-77 cells and Raji cells were collected (3 x 10^6 cells) in RPMI++, spun down (1500 rpm; 5 min), resuspended in 140 µl ^{51}Cr (Chromium-51; CJS11-1mCi, batch 12; 140 µl is about 100 µCi) and incubated

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(37 °C water bath; 1 hour). After washing cells (1500 rpm, 5 min, in PBS, 3x), cells were resuspended in RPMI++ and counted by trypan blue exclusion. Cells were brought at concentration of 2 x 10^4 cells/ml.

Preparation of effector cells: Fresh peripheral blood mononuclear cells (MNC) were isolated from 40 ml of heparin blood by Ficoll (Bio Whittaker; lymphocyte separation medium, cat 17-829E) via manufacturer's instructions. After resuspension of cells in RPMI++, cells were counted by trypan blue exclusion and adjusted to a concentration of 1 x 10^6 cells/ml.

ADCC set up: 50 µl RPMI++ was pipetted into 96 wells plates, and 50 µl of ^{51}Cr-labeled targets cells were added. Thereafter, 50 µl of antibody was added, diluted in RPMI++ (final concentrations 10, 1, 0.1, 0.01 µg/ml). Cells were incubated (RT, 10 min), and 50 µl effector cells were added, resulting in an effector to target ratio of 50:1 (for determination of maximal lysis, 50 µl 5% Triton-X-100 was added instead of effector cells). Cells were spun down (500 rpm, 5 min), and incubated (37 °C, 5% CO_2, 4 hours). After spinning down the cells (1500 rpm, 5 min), 100 µl of supernatant was harvested into micronic tubes, and counted in a gamma counter. The percentage specific lysis was calculated as follows:

% specific lysis = (cpm sample- cpm target cells only)/(cpm maximal lysis –cpm target cells only) x 100

Statistics: Data were analyzed by one-way ANOVA, followed by Tukey's multi comparison post-hoc test. Analysis was performed using Graph Pad Prism (version 3.02 for Windows, Graph Pad Software, San Diego, CA, USA).

Antibody concentration-dependent lysis of ARH-77and Raji cells: 2F2T and 11B8T were tested for their ability to induce ADCC of ARH-77 and Raji cells (n=3) in comparison with rituximab.

A dose-effect relation with CD20 mAbs was observed in ADCC of ARH-77 cells using MNC as effector cells (Figure 27). Both 2F2T and 11B8T induced specific lysis of ARH-77 cells which was maximal (50%) at 10 µg/ml of mAb. Rituximab induced only 25% lysis of target cells. Addition of the isotype control antibody (HuMab-KLH) did not induce ADCC. No specific lysis was observed without addition of MNCs (data not shown).

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When Raji cells were used as target cells, a similar picture as with ARH-77 cells emerged (Figure 28). Both 2F2T and 11B8T induced MNC-mediated lysis of Raji cells, albeit that 2F2T seemed more potent than 11B8T at low concentrations. The maximum lysis reached with 2F2T and 11B8T was approximately 35%. Rituximab induced MNC-mediated lysis of Raji cells, although only 20% of target cells were susceptible to rituximab. Addition of the isotype control antibody (HuMab-KLH) did not induce ADCC. No specific lysis was observed without addition of MNCs (data not shown).

Example 8 FRET and Triton-X insolubility analysis

Preparation of Cy3- and Cy5-conjugated mAb for fluorescence resonance energy transfer (FRET): Monoclonal antibodies were directly conjugated to bifunctional NHS-ester derivatives of Cy3 and Cy5 (Amserham Biosciences UK Ltd) as described in the manufacturer's instructions. Briefly, mAb were dialyzed against 0.1 M carbonate/bicarbonate buffer (pH 9). Thereafter, dye was dissolved in H_2O, immediately added to 1 mg of the mAb, and incubated at room temperature in the dark for 45 min. The labeled mAbs were separated from the unconjugated dye by gel chromatography using a PD10-Sephadex G25 column equilibrated in PBS. Molar ratios of coupling were determined spectrophotometrically from ε_{552} = 150/mM/cm for Cy3, ε_{650} = 250/mM/cm for Cy5, and ε_{280} = 170/mM/cm for protein, and ranged from 5- to 8-fold excess dye:protein.

FRET analysis: Daudi cells were resuspended at 5 x 10^6 cells/ml in PBS/0.1% BSA, and equimolar donor (Cy3)-conjugated and acceptor (Cy5)-conjugated mAb were combined and added to the cell suspension (final concentration 10 µg/ml). Cells were incubated for 30 min in the dark, at 4 °C or 37 °C. Each experiment included cells labeled with donor- and acceptor-conjugated mAb after pre-incubation with a 20-fold molar excess of unconjugated mAb, and cells labeled with donor-or acceptor-conjugated mAb in the presence of equimolar unlabeled mAb. To assess the association of labeled antigens, flow cytrometric FRET measurement was carried out using a FACScalibur (BD Biosciences). The fluorescence intensities at 585 nm (FL2) and 650 nm (FL3), both excited at 488 nm, and the fluorescence intensities at 661 nm (FL4), excited at 635 nm, were detected and used to calculate FRET according to the equitation below, where A is acceptor (Cy5), and D is donor (Cy3). All values obtained were corrected for autofluoresence using the following formula:

FRET = FL3(D,A)-FL2(D,A)/a-FL4(D,A)/b
where a = FL2(D)/FL3(D), and b = FL4(A)/FL3(A)

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Correction parameters were obtained using data collected from single-labeled cells, and side angle light scattering was used to gate out debris and dead cells. FRET between donor and acceptor mAb derivatives on dually labeled cells is expressed in terms of acceptor sensitized emission at 488 nm. Larger FRET values indicate closer physical association of the donor- and acceptor labeled antibodies or a higher density of acceptor-labeled mAb in the vicinity of donor-labeled mAb.

Assessment of raft associated antigen by Triton X-100 (TX) insolubility: As a rapid assessment of the presence of antigen in raft microdomains, a flow cytometry method based on Triton X-100 (TX) insolubility at low temperatures was used, as described previously. In brief, Daudi cells were washed in RPMI/ 1% BSA and resuspended at 2.5 x 10^6/ml. The cells (100 µl) were then incubated with 10 µg/ml of FITC conjugated mAb for 15 min at 37 °C, washed in cold PBS/ 1% BSA/ 20 mM sodium azide (PBS-BS), and the sample was divided in half. All samples were kept on ice throughout the remainder of the assay. One half was maintained on ice to allow calculation of 100% surface antigen levels, whilst the other was treated with 0.5% TX for 15 min on ice to determine to proportion of antigens remaining in the insoluble raft fraction. Cells were then maintained at 4 °C throughout the remainder of the assay, washed once in PBS-BS, resuspended in PBS-BS and assessed by flow cytometry. To determine the constitutive level of raft association of the target antigens, cells were first treated with 0.5% TX for 15 min on ice and washed in PBS-BS prior to binding of FITC-labeled mAb.

As shown in Figures 29A, 29B, and 29, fluorescence resonance energy transfer (FRET) analysis indicates clustering of CD20 upon incubation with 2F2 or 7D8. No such clustering was observed upon incubation with 11B8. These results are consistent with the TX treatment data, cf. Figure 29C, (*i.e.*, 2F2 and 7D8, unlike 11B8 remain with the insoluble fraction of the cell following binding) and support the concept that 2F2 and 7D8, upon binding, translocate CD20 into lipid raft compartment of the B cell membrane.

As shown if Figure 30 (FRET values and s.e.m. of three experiments using one-way ANOVA followed by Tukey's multi comparison post-hoc test) the FRET analysis indicates clustering of rituximab and 2F2, whereas no clustering was observed with 11B8T. These data are in agreement with the data obtained after treatment with 0.5% TX prior to binding of FITC-labeled mAbs as shown in Figure 31 (n=2).

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Preparation of lipid raft fractions and Western blotting: Another way to examine the association of CD20 with lipid rafts, is to investigate the distribution of CD20 between the raft and non-raft membrane fractions using the sucrose gradient fractionation method as disclosed by Deans, J.P., et al., *J. Biol. Chem.*, 1998. **273**(1): pp 344-348, except that Optiprep (Sigma) was used instead of sucrose. Monoclonal antibodies directed against CD20 (10 µg/ml) were allowed to bind to Daudi cells (1 x 10^7) for 20 min at 37 °C. Following this incubation, the cells were pelleted, washed twice with PBS and lysed in ice-cold lysis buffer (1.0% TX in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 10 mM EDTA)). The cell pellet was resuspended thoroughly and incubated for 20 min on ice. Thereafter, the lysate was mixed with 400 µl cold 60% Optiprep (Sigma). The sample was overlaid with a 600 µl step of each 35%, 30%, 25%, 20%, 0% Optiprep in lysis buffer. The gradients were spun at 40.000 rpm at 4 °C for 18 hours. Six fractions from the top were collected, resolved on a 4-15% SDS-PAGE gel, transferred onto nitrocellulose membranes and incubated with primary antibody (mouse anti-CD20 polysera; Serotec, UK), followed by HRP-conjugated secondary antibody (rabbit anti mouse-HRP; Jackson, Bar Harbor, Maine, USA). Blots were visualised using Supersignal West Dura extended duration substrate (Pierce, Woburn, MA, USA).

The results are shown in Figure 32. As it can be seen, CD20 molecules are confined to the high-density fraction 5 (untreated cells). Cells treated with rituximab showed a distinct shift in CD20 distribution with a significant proportion in the lower density membrane fractions 2 and 3, coincident with the fraction where membrane rafts are expected to sediment. Cells treated with 2F2 also showed this shift to fractions 2 and 3. In contrast, cells treated with 11B8T for 20 min showed a similar distribution to untreated cells, with CD20 molecules in fraction 5. In conclusion binding to 2F2 and rituximab induces a shift of CD20 molecules to the lower density membrane fractions, whereas binding to 11B8T does not.

Example 9 Apoptosis of Burkitt Cell Lines with Human Antibodies Against CD20

Apoptosis: Daudi cells, 0.5 x 10^6 in 1 ml tissue culture medium, were placed into 24-well flat-bottom plates with 1 or 10 µg/ml mAb or control antibodies, and incubated at 37 °C. After 20 hours, cells were harvested, washed in Annexin-V-FITC binding buffer (BD biosciences) and labeled with Annexin V-FITC (BD biosciences) for 15 min in the dark at 4 °C. The cells were kept at 4 °C until analysis. Each sample (150 µl) was added to 10 µl of PI solution (10 µg/ml in PBS) in a FACS tube. The mixture was assessed immediately by flow cytometry using a FACScalibur flow cytometer with

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CellQuest pro software (BD Biosciences, Mountain view, CA). At least 10,000 events were collected for analysis.

Induction of apoptosis in Daudi cells: Daudi cells were incubated for 20 hours in the presence of human antibodies against CD20 (1 μg/ml) (without the addition of a secondary cross-linking antibody). Induction of apoptosis was assessed by AnnexinV/PI staining using flow cytometry.

As shown in Figures 33A-G, 11B8 shows clear evidence of inducing apoptosis (similar to that induced by an anti-IgM antibody). 2F2 and 7D8 did not induce apoptosis of Daudi cells. An apoptosis-inducing mouse anti-CD20 antibody, AT80, was used as a control.

Induction of apoptosis in Raji cells: Induction of apoptosis of Raji cells was tested with a concentration range of CD20 mAbs. Figure 34 shows the percentage of annexin-V-positive cells. As can be seen from Figure 34, the positive control mouse anti-human CD20-mAb, B1, induced a concentration-dependent increase in apoptosis of Raji cells with a maximum of approximately 70% at 10 μg/ml mAb. Also 11B8 was a strong inducer of apoptosis, resulting in apoptosis of Raji cells with a maximum of 53.4% at 10 μg/ml mAb. On the other hand, 2F2 and rituximab were very poor in inducing apoptosis of Raji cells, with slightly elevated levels of apoptosis compared to negative control levels.

Induction of apoptosis in Daudi cells: The same picture emerged when Daudi cells were used as target cells, after addition of 1.0 μg/ml CD20 mAb (Figures 35A and 35B). Data in Figure 35A show the total of annexin-V positive cells, and data in Figure 35B (the X-axis showing annexin-V, and the Y-axis showing PI) show the percentages of Daudi cells in early apoptosis (annexin-V positive and PI negative) and late apoptosis (annexin-V positive and PI positive). Again, Both B1 (65.9%) and 11B8T (56.3%) were strong inducers of apoptosis (Figure 36), when used at a concentration of 1.0 μg/ml. Addition of 2F2T resulted in a low level of apoptotic Daudi cells (17%). Addition of rituximab resulted in approximately 29% apoptosis of Daudi cells. Addition of isotype control antibody HuMab-KLH did not induce apoptosis of Daudi cells (6%).

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Example 10 Homotypic Adhesion of Cells with Human Antibodies Against CD20

Homotypic aggregation correlates with induction of apoptosis. Therefore, the ability of the anti-CD20 mAbs to induce homotypic aggregation of B cells was investigated

Homotypic aggregation of Ramos-EHRB cells: Ramos-EHRB cells (0.5 x 10^6 in 1 ml tissue culture medium) were incubated at 37 °C for 4 hours in the presence of anti-CD20 antibodies 11B8, 2F2, or 7D8 (without cross-linking) and induction of homotypic adhesion was assessed by light microscopy (as described above).

As shown in Figures 36A-E, 11B8 caused extensive aggregation of Ramos-EHRB cells (similar to the aggregation caused by murine anti-CD20 antibody, AT80). 2F2, and 7D8 did not induce homotypic aggregation of Ramos cells.

Homotypic aggregation of Daudi cells: Daudi cells were placed into 24-well flat-bottom plates with 1 or 10 µg/ml anti-CD20 mAbs or control antibody, and incubated at 37 °C for 4 hours. The extent of homotypic aggregation was determined by light microscopy. As can be seen from Figure 37, 2F2 hardly induced homotypic aggregation of Daudi cells, with 1.0 µg/ml (and 10 µg/ml, data not shown). Rituximab gave little homotypic aggregation of Daudi cells. In contrast, the B1 antibody was a strong inducer of homotypic aggregation.

Example 11 Immunotherapy Using Human Antibodies Against CD20

Therapy with high dose (100 µg) 2F2 and 7D8 of SCID mice challenged with Daudi cells: The SCID mice were obtained from Harlan UK Ltd., Blackthorn, Oxon, UK, and bred and maintained under pathogen free conditions. Daudi cells (2.5 x 10^6) were injected i.v. into the tail vein of cohorts of 12-16 weeks old SCID mice, followed 7 days later by injection of 100 µg of 2F2 or 7D8 via the same route. Animals were sacrificed upon presentation of limb paralysis, according to the instructions of the animal ethics committee. As shown in Figure 38, survival of the mice is prolonged after treatment with 2F2 or 7D8.

Therapy with high dose (100 µg) 2F2 and rituximab of SCID mice challenged with Tanoue cells: Tanoue cells (2.5 x 10^6 in 200 µl PBS) were injected i.v. into the tail vein of cohorts of 12-16 week old SCID mice (Harlan UK Ltd., Blackthorn, Oxon, UK) followed 7 days later by the injection of 100 µg (in 200 µl PBS) of anti-CD20 mAb via the same route. In this experiment, 2F2 was compared to rituximab and B1. Animals were sacrified upon presentation of rear-limb paralysis. The results are shown in Figure 39. At day 39, the first two control mice died, and death within this

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group was complete at day 54. Only one mouse died within this time interval following 2F2 treatment and survival was considerably increased for the other mice in this group. One mouse died 81 days following injection of the tumor cells and the remaining mice (60% of the total number) survived beyond the termination of the experiment at 100 days post tumor challenge. Rituximab in contrast only increased survival for 2 out of 5 mice (dying at 66 and 83 days post challenge) and none of the mice survived until the end of the experiment. In the B1-group, the survival of SCID mice was similar to that in the 2F2 group, with two mice dying on day 48, and one mouse on day 76. In this group, forty percent was alive at the time the experiment was terminated.

Dose response of 2F2 and rituximab treatment of SCID mice challenged with Daudi cells: To assess the efficacy of 2F2 in comparison to rituximab in protection against tumorigenesis, a dose titration was performed in therapy of SCID mice challenged with Daudi tumor cells. Daudi cells express more CD20 than Tanoue cells and are more sensitive to killing *in vitro*. 10 groups of SCID mice (4 per group) and 1 control group (5 SCID mice) were injected with 2.5 x 10^6 Daudi cells (in 200 µl PBS) i.v. on day 0, and then treated with 20, 5, 2, 0.5 or 0.1 µg (in 200 µl PBS) rituximab, 2F2 or PBS (control) i.v. on day 7. Animals were sacrificed upon presentation of rear-limb paralysis. The results are shown in Figure 40.

In the control group, all mice died within the time interval of 26-29 days. However, a clear dose-effect relation was observed with 2F2 (Figure 40, upper graph). Whereas no effect was observed with doses of 0.1 µg and 0.5 µg 2F2, as little as 2 µg 2F2 substantially extended survival until day 41, 5 µg 2F2 extended survival until day 47, and 20 µg 2F2 extended survival even until day 50.

In contrast, rituximab even tested at the highest dose of 20 µg only slightly increased survival and no dose-effect relation was therefore observed at the lower concentrations tested (Figure 40, lower graph).

Therapy of SCID mice with Daudi tumors by 11B8T and B1: Daudi cells (2.5 x 10^6) in 200 µl PBS were injected i.v. into the tail vein of cohorts of 12-16 week old SCID mice, followed 7 days later by the injection of 100 µg 11B8 or B1 in 200 µl PBS via the same route. Animals were sacrificed upon presentation of rear-limb paralysis. In control mice treated with PBS, all mice died within a time interval of 35-53 days (Figure 41). 11B8T treatment strongly protected the mice, with mice dying between 72 and 98 days post tumor challenge. In the B1-treatment group, most mice survived until day 98 and 40% of the mice survived beyond the end of the experiment, *i.e.*, day 100.

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Example 12 Evaluation of anti-CD20 antibodies in a Daudi-luc xenograft model using SCID mice

The therapeutic efficacy of anti-CD20 antibodies was evaluated in a mouse model in which disseminated outgrowth of human B-cell tumor cells is followed using external optical imaging. In this model tumor cells are transfected with firefly luciferase. Upon administration of luciferin (Molecular Probes, Leiden, The Netherlands) to the mice the labeled cells can be detected in *vivo* by bioluminescent imaging using a highly sensitive CCD camera, cf. Wetterwald *et al.* (2002) *American Journal of Pathology*, 160(3):1143-1153.

Daudi cells were transfected with gWIZ luciferase from Gene Therapy Systems (San Diego, CA) and cultured in RPMI with 10% FCS, Pen/Strep, Sodium Pyruvate and 1 ug/ml puromycin (Sigma). Cells were analysed for luciferase expression (expressed in RLU/1 x 10^5 cells) in a luminometer and for CD20 expression by FACS. 2.5 x 10^6 luciferase-transfected Daudi cells/mouse were injected i.v. into SCID mice. Eight days after inoculation, the mice received a single dose (10 μg) treatment of 2F2T, 11B8T, rituximab, B1 or isotype control antibody (huIgG1) (6 mice per treatment group). For imaging, mice were anesthetized by i.p. injection of a mixture of ketamine /xylazine /atropine. Synthetic D-Luciferin (sodium salt, Molecular Probes) was given i.p. at a dose of 25 mg/ml. Mice were then placed in a light tight box and after 3 min, imaging was started using a VersArray 1300B liquid nitrogen cooled CCD detector (Roper Scientific). Photons emitted from the luciferase were counted over an exposure period of 5 min. Under illumination black and white images were made for reference. MetaVue software (Universal Imaging Corp) was used for data collection and image analysis. Statistical significance of differences between groups was established using one-way analysis of variance with a Newman-Keuls post test using GraphPad PRISM version 3.02 (Graphpad Software Inc).

Imaging from the back side was performed at one-week intervals. On day 8, the day of treatment, light emission was only detected at the inoculation sites in the tail. Tumor formation at distant sites was detected on day 14 in all mice from the isotype control group (huIgG1) and in one mouse from the rituximab group. In the following weeks light emission steadily increased. Figure 42 gives the images of all mice made on day 39 (31 days after treatment), in which bioluminescence is represented in red color (the dark areas in the mice) (light intensity > 50 photons per 5 min) as overlay on the black and white body image of the mice. The tumor mass in each mouse was quantified on day 25, 32, 39, and 46 by integrating the light signals over the body surface, cf. Figure 43. The fastest tumor growth was observed in the isotype control group. Treatment with rituximab gave significant inhibition of tumor growth. However,

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tumor growth inhibition by 2F2T, 11B8T and B1 was significantly more potent (see below Table 2 for significance levels.

Table 2

Significance levels of differences in integrated light intensity between groups at different time points

	Day 25	**Day32**	**Day 39**	**Day 46**
B1 vs. rituximab	$P > 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.001$
B1 vs. 11B8T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
B1 vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
B1 vs. huIgG1	$P < 0.001$	$P < 0.001$	$P < 0.001$	
rituximab vs. 11B8T	$P > 0.05$	$P < 0.05$	$P < 0.01$	$P < 0.001$
rituximab vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P < 0.001$
rituximab vs. huIgG1	$P < 0.001$	$P > 0.05$	$P < 0.05$	
11B8T vs. 2F2T	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
11B8T vs. huIgG1	$P < 0.001$	$P < 0.001$	$P < 0.001$	
2F2T vs. huIgG1	$P < 0.001$	$P < 0.01$	$P < 0.01$	

Example 13 Pilot and Pharmacokinetic study in cynomolgus monkeys

The objective was to determine the pharmacokinetic pattern and pharmacological effects of 2F2 in cynomolgus monkeys (approximately 2 years old; weight range of 2.1-2.6 kg) following once daily intravenous infusion administrations (*via* the saphenous vein) for 4 consecutive days. The study also compared the pharmacological effects of rituximab in order to determine its equivalent potential. For this purpose, 6 male and 6 female cynomolgus monkeys were assigned to 6 dose groups that received 2F2 or rituximab at dose levels of 1.25, 6.25 and 12.5 mg/kg/day at a constant dose volume of 10 ml/kg for 4 consecutive days, in total 5, 25 and 50 mg/kg, respectively. On completion of the last dose administration, the animals were retained for a post dose observation period of 130 days. The practices and procedures adopted during this study were consistent with the OECD Principles of Good Laboratory Practice as set forth by the United Kingdom Department of Health. All animals were observed at regular intervals for signs of ill health or reaction to treatment and were subjected to a physical examination. Laboratory investigations of haematology, coagulation, clinical chemistry and urine analysis were performed during the study. Blood samples and lymph node biopsies were obtained (from the superficial lymph nodes) for flow cytometry analysis throughout the dosing and post dose observation periods. The following cell phenotypes were analysed by flow cytometry: CD3, CD4, CD8, CD20

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and CD21. On completion of the post dose observation period the animals were sacrificed and subjected to a detailed necropsy.

There were no adverse clinical signs or any findings that were considered to be related to treatment with 2F2 or rituximab. Figures 44 and 45 shows the flow cytometry analysis of CD20 and CD21 expressing cells in peripheral blood of treated animals, respectively. Figure 46 shows the flow cytometry analysis of CD20 expressing cells in lymph nodes. Together, both phenotypes analysed during the study indicate a strong and efficient B cell depletion after administration of 2F2 and rituximab at 6.25 mg/kg/day (25 mg/kg in total) and 12.5 mg/kg/day (50 mg/kg in total). In addition, data shows that repopulation of CD20 expressing cells in the lymph nodes and peripheral blood of 2F2 treated animals restarted approximately at day 75 post dosing of 25 mg/kg and 50 mg/kg, *i.e.*, markedly later than in rituximab treated animals.

Furthermore, Figures 47A-C show the flow cytometric analysis of $CD20^{low}CD23^{+}CD40^{high}$ expressing cell subpopulations in the peripheral blood (Y. Vugmeyster *et al.* (2003) Cytometry 52A:101-109).

Peripheral blood cells obtained from either 2F2 or rituximab treated monkeys at dose levels of 1.25 mg/kg (Figure 47A), 6.25 mg/kg (Figure 47B), and 12.5 mg/kg (Figure 47C) once daily by intravenous infusion administrations for 4 consecutive days were incubated with anti-human CD20 FITC murine monoclonal antibody (Coulter) at room temperature for 10 min. Afterwards, count beads were added together with PBS and the cells were washed twice (300 g for 10 min), followed by immediate analysis of $CD20^{low}CD23^{+}CD40^{high}$ vs. $CD20^{high}$ $CD23^{+}CD40^{low}$ expressing cell subpopulation in a flow cytometer (Beckman Coulter). Results of $CD20^{low}$ $CD23^{+}CD40^{high}$ cells shown are expressed as cells per μl. As can be seen from the Figure 47 2F2 was capable of inducing a complete and longer depletion of $CD20^{low}CD23^{+}CD40^{high}$ expressing cells compared to rituximab.

Example 14 Epitope mapping using site-directed mutagenesis

Epitope mapping studies using a mutagenesis approach have indicated that alanine at position 170 (A170) and proline at position 172 (P172) in the second extracellular loop are critical for the recognition of human CD20 by known anti-CD20 antibodies. In studies by Deans and colleagues (M.J. Polyak, *et al.*, *Blood*, (2002) 99(9): pp 3256-3262; M.J. Polyak, *et al.*, *J. Immunol.*, (1998) 161(7): pp 3242-3248) the binding of all anti-CD20 mAbs tested was abrogated by changing A170 and P172 into the corresponding murine CD20 residues S170 and S172. Some heterogeneity in the recognition of the AxP epitope has been recognized however as most antibodies like rituximab recognize murine CD20 with S170 and S172 mutated to the human A170xP172 sequence whereas some others require additional mutations immediately N-

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terminal of the AxP sequence. To verify whether the A170xP172 motive is also important for the binding of the antibodies according to the invention the AxP sequence was mutated into SxS using site-directed mutagenesis (AxP mutant = A170S, P172S), cells were transfected with the AxP mutant and wild-type (WT) CD20 DNA, and the binding characteristics of the anti-CD20 mAbs were compared.

Further mutants were prepared, P172S (proline at position 172 mutated to serine), N166D (asparagine at position 166 mutated to aspartic acid), and N163D (asparagine at position 163 mutated to aspartic acid), using site-directed mutagenesis to evaluate whether the mutated amino acid residues are important for binding of the antibodies of the invention.

To examine this, a CD20 expression vector was constructed by amplifying the CD20 coding sequence using suitable primers introducing restriction sites and an ideal Kozak sequence for optimal expression. The amplified fragment was digested and ligated in the expression vector pEE13.4. After transformation in *E. coli*, colonies were screened for inserts and two clones were selected for sequencing to confirm the correct sequence. The construct was named pEE13.4CD20HS.

Mutagenesis was performed to introduce the AxP mutation and to introduce 20 mouse mutations in the extracellular loop regions of human CD20. Mutagenesis was checked by restriction enzyme digestion and sequencing. The constructs were transiently transfected in CHO cells (for AxP mutations) or HEK293F cells and analyzed 24 or 48 hours post-transfection using flow cytometry.

Oligonucleotide PCR Primers: Oligonucleotide primers were synthesized and quantified by Isogen BV (Maarssen, The Netherlands). Primers were reconstituted in water in a concentration of 100 pmol/µl and stored at -20 °C until required. A summary of PCR and sequencing primers is shown in Table 3.

Optical density determination of nucleic acids: Optical density was determined using an Ultrospec 2100 pro Classic (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The DNA concentration was measured by analysis of the OD_{260nm}, where one OD_{260nm} unit = 50 µg/ml. The reference solution was identical to the solution used to dissolve the nucleic acids.

Plasmid DNA isolation from E. coli culture: Plasmid DNA was isolated from *E. coli* cultures using kits from Qiagen according to the manufacturer's instructions (Westburg BV, Leusden, The Netherlands). For 'bulk' plasmid preparation either a Hi-Speed plasmid Maxi kit or a Hi-Speed plasmid Midi kit were used (Qiagen). For a small scale plasmid preparation (*i.e.*, 2 ml of *E coli* culture) a Qiaprep Spin Miniprep Kit

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(Qiagen) was used and the DNA eluted in 50 µl TE (Tris-HCl 10 mM pH 8.0, EDTA 1 mM).

PCR amplification: PCR reactions were performed according to the manufacturer's instructions for the Pfu-Turbo© Hotstart DNA polymerase (Stratagene, Amsterdam, The Netherlands). Each 20 µl reaction contained 1 x PCR reaction buffer, 200 µM mixed dNTPs, 6.7 pmol of each forward and reverse primer, approximately 1 ng template DNA and 1 unit of Pfu-Turbo© Hotstart DNA polymerase. PCR reactions were performed on a T-gradient Thermocycler 96 (Biometra GmbH, Goettingen, Germany) using a 30 cycle program of: +95 °C for 2 min, followed by 30 cycles of: +95 °C for 30 sec, anneal: a gradient of 45-65 °C for 30 sec and extension: +72 °C for 2 min, followed by a final extension step of 10 min at 72 °C and subsequent storage at 4 °C. The completed reactions were analysed by agarose gel electrophoresis.

Agarose gel electrophoresis: Agarose gel electrophoresis was performed according to Sambrook (Molecular Cloning Laboratory Manual, 3rd edition) using gels of 50 ml, in 1 x Tris/acetic acid/EDTA (TAE) buffer. DNA was visualized by the inclusion of ethidium bromide in the gel and observation under UV light. Gel images were recorded by a CCD camera and an image analysis system (GeneGnome; Syngene, Cambridge, UK).

Restriction enzyme digestions: Restriction enzymes were supplied by New England Biolabs (Beverly, MA) and used according to the supplier's recommendations. In general, 100 ng was digested with 5 units of enzyme(s) in appropriate buffer in a final volume of 10 µl. Reaction volumes were scaled up as appropriate. Digestions were incubated for a minimum of 60 min at the manufacturer's recommended temperature.

For fragments requiring double digestions with restriction enzymes which have incompatible buffer or temperature requirements, digestions were performed sequentially so as to offer favourable conditions for each enzyme in turn.

Alkaline phosphatase treatment: Shrimp alkaline phosphatase (USB, Cleveland, OH) was used according to the supplier's recommendations. Alkaline phosphatase removes 5'-phosphate groups from the ends of DNA fragments thereby preventing self-ligation. This is of particular relevance when self re-ligation of a DNA fragment could result in a replication-competent vector. The enzyme is active in most restriction enzyme buffers and was added as appropriate. After the digestion, the enzyme was inactivated by raising the temperature to 70 °C for 15 min.

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Purification of PCR and restriction enzyme reaction products: Purification was carried out using the mini-elute PCR Purification kit (supplied by Qiagen), according to the manufacturer's instructions. Briefly, DNA samples were diluted in 5 volumes of binding buffer I (Qiagen) and loaded onto a mini-elute column within an Eppendorf centrifuge tube. The assembly was centrifuged in a bench-top microcentrifuge. The column was washed twice with buffer II (Qiagen): Following buffer application, the assembly was centrifuged and the flow-through was discarded. The column was dried by centrifugation in the absence of added buffer. DNA was eluted by adding elution buffer to the column and the eluate collected by centrifugation. Isolated DNA was quantified by UV spectroscopy and quality assessed by agarose gel electrophoresis.

Isolation of DNA fragments from agarose gel: Where appropriate (*i.e.*, when multiple fragments were present), digested DNA samples were separated by gel electrophoresis and the desired fragment excised from the gel and recovered using the QIAEX II gel extraction kit (Qiagen), according to the manufacturer's instructions. Briefly, DNA bands were excised from the agarose gel and melted in an appropriate buffer at +55 °C. QIAEX II resin was added and incubated for 5 min. QIAEX II resin was pelleted by a short centrifugation step (1 min, 14000 g, RT) and washed twice with 500 µl of wash buffer PE. The final pellet was dried in a hood and DNA was eluted with the appropriate volume of TE and temperature (depending on the size of the DNA).

Ligation of DNA fragments: Ligations were performed with the Quick Ligation Kit (New England Biolabs) according to the manufacturer's instructions. For each ligation, the vector DNA was mixed with approximately 3-fold molar excess of insert DNA such that the total amount of DNA was lower than 200 ng in 10 µl, with volume adjusted with water as appropriate. To this was added 10 µl 2 x Quick Ligation Buffer and 1 µl Quick T4 DNA ligase and the ligation mix was incubated for 5-30 min at room temperature.

Transformation of DNA into bacteria: Samples of DNA were used to transform One Shot DH5α-T1R competent *E. coli* cells (Invitrogen, Breda, The Netherlands) using the heat-shock method according to the manufacturer's instructions. Briefly, 1-5 µl of DNA solution (typically 2 µl of DNA ligation mix) was added to an aliquot of transformation-competent bacterial cells and the mixture incubated on ice for 30 min. The cells were then heat-shocked by transferring to a waterbath at 42 °C for 30 sec followed by a further incubation on ice for 5 min. Cells were left to recover by

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incubation in a non-selective culture medium (SOC) for 1 hour with agitation at 37 °C and were subsequently spread onto agar plates containing appropriate selective agent (ampicillin at 50 µg/ml). Plates were incubated for 16-18 hours at +37 °C or until colonies of bacteria became evident.

Screening of bacterial colonies by PCR: Bacterial colonies were screened for the presence of vectors containing the desired sequences using the PCR colony screening technique. 20 µl of PCR reaction mix containing 0.5 volumes of HotStarTaq Master Mix (Qiagen), 4 pmol of the forward and reverse primers and completed with water was added to a PCR tube. A colony was lightly touched with a 20 µl pipet tip, once touched in 2 ml LB in a culture tube (for growing bacteria containing the corresponding plasmid) and resuspended in the 20 µl PCR mix. PCR was performed on a T-gradient Thermocycler 96 (Biometra) using a 35 cycle program of: +95 °C for 15 min, followed by 35 cycles of: +94 °C for 30 sec, anneal: 55 °C for 30 sec and extension: +72 °C for 2 min, followed by a final extension step of 10 min at 72 °C and subsequent storage at 4°C. The completed reactions were analyzed by agarose gel electrophoresis. See Table 3 for details of primer pairs used for colony PCR.

DNA sequencing: Plasmid DNA samples were send to AGOWA (Berlin, Germany) for sequence analysis. Sequences were analyzed using the VectorNTI software package (Informax, Frederick, MD, USA).

Table 3

Name	Application	Length	Oligo Sequence
CD20P172S	CD20 mutagenesis	36	TGGGGAGTTTTTCTCAGAGGAATTCGATGGTTCACAGTTGTA
CD20N166D	CD20 mutagenesis	39	TGTAACAGTATTGGGTAGATGGG
CD20N163D	CD20 mutagenesis	36	AATCATGGACATACTTAATATTA
cd20exfor	CD20 construction	41	TATAGCCCGGGGCCGCCACCATGACAACACCCAGAAATTCA
cd20exrev	CD20 construction	38	GCGTCTCATGTACATTAAGGAGAGCTGTCATTTTCTAT
pee13.4seqrev2	Colony PCR	23	TCGGACATCTCATGACTTTCTTT
pConKseq1	Colony PCR	23	GTAGTCTGAGCAGTACTCGTTGC
cd20hsapmutr (AxP)	CD20 mutagenesis	42	TGGGGAGTTTTTCTCAGAGGAATTCGATGGTTCACAGTTGTA
cd20hsapmutf (AxP)	CD20 mutagenesis	42	TACAACTGTGAACCATCGAATTCCTCTGAGAAAAACTCCCCA
CD20seq2	CD20 sequencing	23	TGTAACAGTATTGGGTAGATGGG
cd20seq1	CD20 sequencing	23	AATCATGGACATACTTAATATTA

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Mutagenesis: The mutagenesis was performed, using either the QuikChange® XL Site-Directed Mutagenesis kit (Cat 200517-5, Lot 1120630, Stratagene Europe) according to the manufacturer's instructions.

Mutagenesis reactions were concentrated using ethanol precipitation and transformed into either oneshot DH5α-T1R competent *E. coli* cells or electroporated into ElectroTen-Blue® Electroporation-Competent Cells. Colonies were checked by colony PCR and restriction digestion prior to transfection.

HEK293F cell transfection: HEK293F cells were obtained from Invitrogen and transfected according to the manufacturer's instructions, using 293fectin. The HEK293F cells were used for all the single mutant sequences.

CHO cell transfection: CHO cells grown to approximately 95% confluence were transiently transfected with CD20 wild-type, mutant cDNA or a combination of both constructs using lipofectamine 2000 (M668-019, Invitrogen, Breda, Netherlands). To this end, 24 µg precipitated DNA was diluted (1µg/µl) in 500 µl optimem, in ratios of AxP 100% : WT 0%; AxP 33.3% : WT 66.6%; AxP 66.6% : WT 33.3%;AxP 0% :WT 100%. For each transfection 24 µl lipofectamine was diluted in 500 µl optimem. Then, the diluted lipofectamine was incubated (RT, 5 min), and the diluted DNA combined with the diluted lipofectamine. After gently mixing and incubating the solution (RT, 20 min), 1000 µl DNA/lipofectamine was added to the CHO cells, thoroughly mixed and incubated for 48 hours at 37 °C, 5% CO_2. Two days after transfection of CHO cells, cells were washed twice with FACS buffer (PBS supplemented with 0.1% BSA and 0.002% NaN_3). CHO cells were treated with trypsin/EDTA (Gibco BRL, Life Technologies, Paisley, Scotland) and lifted off the culture plates.

Anti-CD20 Antibody binding: HEK293F cells and CHO cells were taken up in PBS in a concentration of 2 x 10^6/ml, and added to round bottom plates (1 x 10^5/well). Then, 50 µl CD20 mAb was added, in serial dilutions of 10, 5, 2.5, or 0 µg per well (4 °C, 30 min). After washing in FACS buffer (PBS supplemented with 0.1% BSA and 0.002% NaN_3), the cells were analyzed on a flow cytometer (Becton Dickinson, San Diego, CA, USA), and 5,000 events per sample were acquired at high flow rate.

As can be seen from Figures 48A-E, all anti-CD20 mAbs bound efficiently to CHO cells expressing WT CD20. As expected, rituximab did not bind the AxP mutant (Figure 48A), and B1 bound this mutant poorly (Figure 48D). Both 2F2 and 11B8 in contrast bound to WT and AxP mutant CD20 equally well (Figure 48B and

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Figure 48C). Titrating the amount of WT CD20 on the surface indeed titrated the binding of rituximab and B1. Both 2F2 and 11B8 again were insensitive to the absence or presence of the mutation.

This study indicates that the binding of 2F2 and 11B8 to human CD20 is insensitive to mutations at amino acid positions 170 and 172. 2F2 and 11B8 therefore represent a new class of CD20 mAbs recognizing a novel CD20 epitope.

Figure 49A shows percentage binding of 2F2, 11B8T, B1 or rituximab to mutant P172S vs. WT CD20, Figure 49B shows percentage binding of 2F2T, 11B8T, B1, CAT (CAT 13.6E12, a mouse monoclonal IgG2A anti-CD20 antibody, Diatec.Com), a control isotype antibody (KLH), or rituximab to mutant CD20 (AxP) vs. WT CD20.

For the mutant wherein asparagine at position 166 has been replaced with aspartic acid (CD20N166D) 2F2 showed very low binding, whereas B1, rituximab and 11B8T were able to bind, see Figure 49C. In a similar experiment CAT 13.6E12 and rituximab were able to bind to CD20N166D, whereas 2F2T only showed very low binding, see Figure 49D. For the mutant wherein asparagine at position 163 has been replaced by aspartic acid (CD20N163D) again rituximab, 11B8T, and B1 were able to bind to CD20N163D, whereas 2F2 and 2F2T only showed very low binding, see Figure 49E. In a similar experiment CAT 13.6E12 and rituximab were able to bind to CD20N163D, whereas 2F2T only showed very low binding, see Figure 49F.

These experiments indicate that 2F2 and 11B8 bind to different epitopes.

Example 15 Epitope mapping using Pepscan method

Synthesis of peptides: 7-, 9-, and 15-mer peptides were synthesized according to standard methods. In some cases chemical linkage of the legs of a 15-mer peptide helps to identify amino acid sequences of a potentially discontinuous epitope. According to known procedures (H.M. Geysen *et al.* (1984) *Proc. Natl. Acad. Sci. USA,* 81:3998; J.W. Slootstra *et al.* (1996) *Mol. Divers.* 1:87; and WO 01/60769), 7-, 9-, and 15-mer peptides were synthesized that could be possible binding sites or epitopes involved in binding of 2F2 or 11B8 to the human CD20 molecule. The 9- and 15-mers were synthesized as loops and screened using credit-card format mini-PEPSCAN cards (455 peptide format/card). In all looped peptides amino acids at varied positions were replaced by a cysteine (*e.g.*, acetyl-XCXXXXXXXXXXXXCX-minicard). The peptides were synthesized using standard Fmoc-chemistry and deprotected using TFA with scavengers. Subsequently, the deprotected peptides were reacted on the microarray with an 0.5 mM solution of 1,3-bis(bromomethyl)benzene in ammonium bicarbonate (20 mM, pH 7.9), supplemented with acetonitrile (1:1 (v/v)). The microarrays were gently shaken in the solution for 30-60 min, while completely covered in the solution. Finally,

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the microarrays were washed extensively with excess of Millipore H_2O and sonicated in disrupt-buffer containing 1% sodium dodecylsulfate, 0.1% β-mercaptoethanol, in PBS (pH 7.2) at 70 °C for 30 min, followed by sonication in millipore H_2O for another 45 min. Subsequently, the microwells were ready for screening in an ELISA-assay.

Pepscan ELISA-assay: The 455-well credit card-format polyethylene cards, containing the covalently linked peptides, were incubated with serum (diluted 1:1000 in blocking solution which contains 5% horse serum (v/v) and 5% ovalbumin (w/v)) (4 °C, over night). After washing, the peptides were incubated with anti-human antibody peroxidase (dilution 1:1000, 1 hour, 25 °C), and after washing the peroxidase substrate, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate and 2 µl/ml 3% H_2O_2 were added. After one hour, the color development was measured. The color development of the ELISA was quantified with a CCD-camera and an image processing system. The set up consists of a CCD-camera and a 55 mm lens (Sony CCD Video Camera XC-77RR, Nikon micro-nikk or 55 mm f/2.8 lens), a camera adaptor (Sony Camera adaptor DC-77RR) and the Image Processing Software package Optimas, version 6.5 (Media Cybernetics, Silver Spring, MD 20910, U.S.A.). Optimas runs on a pentium II computer system.

The absorbances (OD values) for the peptides at different antibody concentrations are shown in below Table 4 and Table 5.

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GMI-055PC

Table 4

	11B8 10 μg/ml	11B8 100 μg/ml	7D8 10 μg/ml	7D8 100 μg/ml	rituximab 10 μg/ml	2F2 10 μg/ml	2F2 100 μg/ml	B1 10 μg/ml	B1 100 μg/ml
KMECLNFIRAHCPYI	**763**	**2997**	134	41	90	48	66	147	304
LKMECLNFIRCHTPY	165	738	160	41	120	49	87	179	216
KMESCNFIRACTPYI	**625**	**3090**	142	52	123	39	78	170	308
MESLCFIRAHCPYIN	179	956	127	55	102	41	65	119	178
CFIRAHTPC	188	534	181	69	134	91	114	170	212
CIRAHTPYC	151	449	186	60	132	57	92	151	195
CRAHTPYIC	**427**	**1605**	188	64	145	48	87	179	216
CAHTPYINC	179	452	174	65	125	42	106	161	172
IPAGIYA	217	950	164	76	177	48	85	165	192
PAGIYAP	**449**	**2501**	170	64	111	43	85	165	300
AGIYAPI	**251**	**2207**	188	73	110	44	98	187	143
GIYAPIC	99	251	152	64	141	34	93	177	147
IYAPICV	137	313	174	58	159	58	99	175	90
GIYAPIA	172	857	177	96	156	62	96	165	121
IYAPIAV	161	654	181	58	116	62	76	161	106

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Table 5

	11B8 10 μg/ml	7D8 10 μg/ml	rituximab 10 μg/ml	2F2 10 μg/ml
PCINIYNAEPANPCE	118	163	152	65
YCNIYNAEPANPSCK	287	181	**2418**	86
ICIYNAEPANPSECN	138	192	142	78
NCYNAEPANPSEKCS	93	121	**2649**	49
ICNAEPANPSEKNCP	115	165	**3283**	43
YCAEPANPSEKNSCS	106	188	**3770**	65
NCEPANPSEKNSPCT	159	183	**3476**	61
ACPANPSEKNSPSCQ	146	148	**250**	77
ECANPSEKNSPSTCY	134	179	188	68

As appears from Table 4, 11B8 showed binding to AGIYAP of the small first extracellular loop of human CD20 at both 10 μg/ml and 100 μg/ml, whereas the other antibodies tested did not show significant binding to AGIYAP.

Furthermore, 11B8 showed binding to MESLNFIRAHTPYI of the second extracellular loop of human CD20 at both 10 μg/ml and 100 μg/ml, whereas the other antibodies tested did not show significant binding to MESLNFIRAHTPYI.

As appears from Table 5, rituximab showed binding to EPANPSEK of the second extracellular loop of human CD20 at both **1** μg/ml and 10 μg/ml, whereas the other antibodies tested did not show significant binding to EPANPSEK.

Example 16 Anti-idiotypic antibodies

Generation of anti-idiotypic antibodies: Mouse anti-idiotypic antibodies were made by immunizing Balb/C mice with 2F2 or 11B8T, and generating hybridomas from spleens of these mice by fusion with NS1 myeloma cells using standard techniques. The following anti-idiotypic antibodies were generated: anti-2F2 sab 1.1, anti-2F2 sab 1.2, anti-2F2 sab 1.3, anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6. These were tested for specific binding to 2F2T, 7D8 and 11B8T. ELISA plates were coated with purified 2F2T, 7D8 or 11B8T (diluted in PBS to a final concentration of 1-2 μg/ml, 37 °C, 2 hours). Plates were blocked with PBS containing 0.05% Tween-20 and 2% chicken serum (RT, 1 hour). Subsequently, the plates were incubated with supernatants from cultures of the anti-idiotypic antibodies (final concentration adjusted to 1-10 μg/ml, RT, 2 hours). Bound mouse anti-idiotypic antibodies were detected with rabbit-anti-mouse IgG-HRP conjugated antibody (Jackson ImmunoResearch).

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As shown in Figure 50 anti-2F2 sab 1.1, anti-2F2 sab 1.2, and anti-2F2 sab 1.3 bind to 2F2T and 7D8, but not to 11B8T or an unrelated, isotype control human antibody. Since 2F2T and 7D8 are very homologous in V_L and V_H sequence, reaction of anti-2F2 idiotypic antibodies with 7D8 was expected.

Figure 51 shows that anti-11B8T sab 2.2, anti-11B8T sab 2.3, anti-11B8T sab 2.4, anti-11B8T sab 2.5, and anti-11B8T sab 2.6 all bind to 11B8T to a similar extent.

Anti-idiotypic antibodies as an immunodiagnostic tool: The 2F2/7D8 and 11B8T specific anti-idiotypic antibodies can be used as an immunodiagnostic tool to detect and quantify levels of human monoclonal antibodies against CD20 in laboratory or patient samples. This may be useful for examining pharmakokinetics of the anti-CD20 antibody or for determining and adjusting the dosage of the anti-CD20 antibody and for monitoring the disease and the effect of treatment in a patient. As an example of such an assay, ELISA plates were coated with 4 μg/ml anti-2F2 sab 1.1, anti-2F2 sab 1.2 or anti-2F2 sab 1.3. Plates were blocked with PBS containing 0.05% Tween-20 and 2% chicken serum (RT, 1 hour). Subsequently, the plates were incubated with a serial dilution of 2F2T (10,000-9.77 ng/ml, RT, 2 hours). Bound 2F2T was detected with mouse-anti-human IgG HRP-conjugated antibody. As shown in Figures 52A-C a dose dependent binding of 2F2T was observed.

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Any combination of the embodiments disclosed in the dependent claims are also contemplated to be within the scope of the invention.

Incorporation by Reference

All patents, pending patent applications and other publications cited herein are hereby incorporated by reference in their entirety.

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We claim:

1. An isolated human monoclonal antibody which binds to human CD20.

2. The antibody of claim 1, selected from the group consisting of an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, a secretory IgA, an IgD, and an IgE antibody.

3. The antibody of claim 2, wherein the antibody is an IgG1 antibody.

4. The antibody of claim 2, wherein the antibody is an IgG3 antibody.

5. The antibody of claim 2, wherein the antibody is an IgG4 antibody.

6. The antibody of claim 2, wherein the antibody is an IgA1 or IgA2 antibody.

7. The antibody of any one of the preceding claims, wherein the antibody dissociates from human CD20 with a dissociation rate constant (k_d) of about 10^{-5} sec^{-1} or less.

8. The antibody of any one of the preceding claims, wherein the antibody binds to human CD20 with an affinity constant (K_D) of about 5 nM or less.

9. The antibody of any one of the preceding claims, wherein the antibody has one or more of the characteristics selected from the group consisting of:

(i) capable of inducing complement dependent cytotoxicity (CDC) of cells expressing CD20 in the presence of complement;

(ii) capable of inducing complement dependent cytotoxicity (CDC) of cells expressing CD20 and high levels of CD55 and/or CD59 in the presence of complement;

(iii) capable of inducing apoptosis of cells expressing CD20;

(iv) capable of inducing antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20 in the presence of effector cells;

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(v) capable of inducing homotypic adhesion of cells which express CD20;

(vi) capable of translocating into lipid rafts upon binding to CD20;

(vii) capable of prolonging the survival of a subject having tumor cells which express CD20;

(viii) capable of depleting cells expressing CD20; and

(ix) capable of depleting cells expressing low levels of CD20 ($CD20^{low}$ cells).

10. The antibody of claim 9, which has one or more of the following characteristics selected from the group consisting of:

(i) capable of inducing at least 20%, and preferably at least 30% CDC mediated lysis of B-CLL cells in the presence of 33 vol/vol% plasma within 3 hours at 37 °C at an antibody concentration of 10 μg/ml;

(ii) capable of inducing at least 20%, and preferably at least 30% lysis of B-CLL cells in the presence of 33 vol/vol% whole blood cells within 3 hours at 37 °C at an antibody concentration of 10 μg/ml;

(iii) capable of prolonging the 50% survival rate of SCID mice injected with Daudi cells by more than 30% at a dose of 20 μg; and

(iv) capable of depleting peripheral B cells expressing low levels of CD20 ($CD20^{low}$ B cells) to undetectable levels for more than 50 days in cynomolgus monkeys at a dosage of 6.25 mg/kg per day for 4 consecutive days.

11. The antibody of claim 10, which is capable of capable of inducing at least 20%, and preferably at least 30% CDC mediated lysis of B-CLL cells in the presence of 33 vol/vol% plasma within 3 hours at 37 °C at an anti-CD20 antibody concentration of 10 μg/ml.

12. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:1 and SEQ ID NO:3, respectively, and conservative sequence modifications thereof.

13. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:5 and SEQ ID NO:7, respectively, and conservative sequence modifications thereof.

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14. The antibody of any of the preceding claims 1-11 encoded by human heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NO:9 and SEQ ID NO:11, respectively, and conservative sequence modifications thereof.

15. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively, and conservative sequence modifications thereof.

16. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:2 and SEQ ID NO:4, respectively.

17. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:6 and SEQ ID NO:8, respectively, and conservative sequence modifications thereof.

18. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:6 and SEQ ID NO:8, respectively.

19. The antibody of any of the preceding claims 1-11 having a human heavy chain and human kappa light chain variable regions comprising the amino acid sequences as set forth in SEQ ID NO:10 and SEQ ID NO:12, respectively, and conservative sequence modifications thereof.

20. The antibody of any of the preceding claims 1-11 having human heavy chain and human kappa light chain variable regions which are at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to the amino acid sequences as set forth in SEQ ID NO:10 and SEQ ID NO:12, respectively.

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21. The antibody of any of the preceding claims 1-11 comprising at least one human variable region selected from the group consisting of:

(i) SEQ ID NOs:2, 4, 6, 8, 10, or 12; and

(ii) a sequence which is at least 90% homologous, preferably at least 95% homologous, and more preferably at least 98%, or at least 99% homologous to any one of the amino acid sequences as set forth in (i) above.

22. An isolated human monoclonal antibody which binds to an epitope on human CD20 defined by the antibody of any one of claims 12-21.

23. An isolated human monoclonal antibody which binds to an epitope on CD20

(i) which does not comprise or require the amino acid residue proline at position 172;

(ii) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172;

(iii) which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166;

(iv) which does not comprise or require the amino acid residue proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166; or

(v) which does not comprise or require the amino acid residues alanine at position 170 or proline at position 172, but which comprises or requires the amino acid residues asparagine at position 163 and asparagine at position 166.

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24. An isolated human monoclonal antibody which binds to CD20, wherein the antibody has one or more of the following characteristics:

(i) binds to mutant P172S CD20 (proline at position 172 mutated to serine) with at least the same affinity as to human CD20;

(ii) binds to mutant AxP (alanine at position 170 mutated to serine, and proline at position 172 mutated to serine) with at least the same affinity as to human CD20;

(iii) shows a reduced binding of 50% or more to mutant N166D (asparagine at position 166 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml; or

(iv) shows a reduced binding of 50% or more to mutant N163D (asparagine at position 163 mutated to aspartic acid) compared to human CD20 at an antibody concentration of 10 μg/ml.

25. An isolated human monoclonal antibody which binds to an epitope in the small first extracellular loop of human CD20.

26. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20.

27. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20, wherein the epitope comprises part of the first small extracellular loop and part of the second extracellular loop.

28. An isolated human monoclonal antibody which binds to a discontinuous epitope on CD20, wherein the epitope has residues AGIYAP of the small first extracellular loop and residues MESLNFIRAHTPYI of the second extracellular loop.

29. An isolated human monoclonal antibody which has the binding characteristics of the antibody of any one of claims 11-28.

30. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 13, 14, 15, 16, 17, or 18;

(ii) conservative sequence modifications of the sequences listed in (i); and

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(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

31. The antibody of claim 30, comprising SEQ ID NO:15.

32. The antibody of claim 30, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 13, 14, 15, 16, 17, or 18;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

33. The antibody of claim 30, comprising SEQ ID NOs: 13, 14, 15, 16, 17, and 18.

34. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 19, 20, 21, 22, 23, or 24;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

35. The antibody of claim 34, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 19, 20, 21, 22, 23, or 24;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

36. The antibody of claim 34, comprising SEQ ID NOs: 19, 20, 21, 22, 23, and 24.

37. An isolated human monoclonal antibody which binds to human CD20 comprising at least one CDR sequence selected from the group consisting of:

(i) SEQ ID NOs: 25, 26, 27, 28, 29, or 30;

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(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

38. The antibody of claim 37, comprising SEQ ID NO:27.

39. The antibody of claim 37, comprising at least four CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 25, 26, 27, 28, 29, or 30;

(ii) conservative sequence modifications of the sequences listed in (i); and

(iii) fragments of any one of the sequences defined in (i) or (ii) which retain the ability to bind to human CD20.

40. The antibody of claim 39, comprising SEQ ID NOs: 25, 26, 27, 28, 29, and 30.

41. The antibody of any of the preceding claims 1-11 comprising at least one CDR selected from the group consisting of

(i) SEQ ID NOs:13, 19, or 25, or a sequence having 1 amino acid substitution, deletion or addition of the sequence of SEQ ID NOs:13, 19 or 25;

(ii) SEQ ID NOs:14, 20, or 26, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:14, 20, or 26;

(iii) SEQ ID NOs:15, or 27, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27;

(iv) SEQ ID NO: 16, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:16;

(v) SEQ ID NO: 17, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:17; and

(vi) SEQ ID NOs: 18, or 30, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs: 18, or 30.

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42. The antibody of claim 41 comprising at least four CDRs selected from the group consisting of

(i) SEQ ID NOs:13, 19, or 25, or a sequence having 1 amino acid substitution, deletion or addition of the sequence of SEQ ID NOs:13, 19 or 25;

(ii) SEQ ID NOs:14, 20, or 26, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:14, 20, or 26;

(iii) SEQ ID NOs:15, or 27, or a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27;

(iv) SEQ ID NO: 16, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:16;

(v) SEQ ID NO: 17, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NO:17; and

(vi) SEQ ID NOs: 18, or 30, or a sequence having 1-2 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs: 18, or 30.

43. The antibody of claim 41 comprising one CDR selected from the group consisting of

SEQ ID NOs:15, or 27, and a sequence having 1-4 amino acid substitutions, deletions or additions of the sequence of SEQ ID NOs:15, or 27.

44. The antibody of any one of the preceding claims which is an intact antibody selected from the group consisting of: an intact IgG1 antibody, an intact IgG2 antibody, an intact IgG3 antibody, an intact IgG4 antibody, an intact IgM antibody, an intact IgA1 antibody, an intact IgA2 antibody, an intact secretory IgA antibody, an intact IgD antibody, and an intact IgE antibody, wherein the antibody is glycosylated in a eukaryotic cell.

45. The antibody of any one of the preceding claims which is an antibody fragment or a single chain antibody.

46. The antibody of any one of the preceding claims which is a binding-domain immunoglobulin fusion protein comprising (i) a binding domain polypeptide in the form of a heavy chain variable region or a light chain variable region as defined in claim 24 that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region.

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47. The antibody of any one of the preceding claims produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, in which V-(D)-J gene segment rearrangements have resulted in the formation of a functional human heavy chain transgene and a functional human light chain transgene, fused to an immortalized cell.

48. A hybridoma comprising a B cell obtained from a transgenic non-human animal in which V-(D)-J gene segment rearrangements have resulted in the formation of a functional human heavy chain transgene and a functional light chain transgene fused to an immortalized cell, wherein the hybridoma produces a detectable amount of the monoclonal antibody of any one of the preceding claims.

49. A hybridoma which produces a human monoclonal antibody encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof.

50. A hybridoma which produces a human monoclonal having IgG heavy chain and kappa light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs: 4, 8, or 12, respectively, and conservative sequence modifications thereof.

51. The antibody of any one of claims 1-45 produced by a transfectoma comprising nucleic acids encoding a human heavy chain and a human light chain.

52. A transfectoma comprising nucleic acids encoding a human heavy chain and a human light chain, wherein the transfectoma produces a detectable amount of the antibody of any one of claims 1-45.

53. A transfectoma which produces a human monoclonal antibody encoded by human IgG heavy chain and human kappa light chain nucleic acids comprising nucleotide sequences in their variable regions as set forth in SEQ ID NOs:1, 5, or 9 and SEQ ID NOs:3, 7, or 11, respectively, and conservative sequence modifications thereof.

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54. A transfectoma which produces a human monoclonal antibody having IgG heavy chain and kappa light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

55. A eukaryotic or prokaryotic host cell which produces a human monoclonal antibody having heavy chain and light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

56. A transgenic non-human animal or plant which produces a human monoclonal antibody having heavy chain and light chain variable regions which comprise the amino acid sequences as set forth in SEQ ID NOs:2, 6, or 10 and SEQ ID NOs:4, 8, or 12, respectively, and conservative sequence modifications thereof.

57. A method of producing a human monoclonal antibody which binds to human CD20, comprising:

immunizing a transgenic non-human animal having a genome comprising a human heavy chain transgene and a human light chain transgene with human CD20 or a cell expressing human CD20, such that antibodies are produced by B cells of the animal;

isolating B cells of the animal;

fusing the B cells with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies specific for human CD20; and

isolating the human monoclonal antibodies specific for CD20 from the culture supernatant of the hybridoma, or the transfectoma derived from such hybridoma.

58. A method according to claim 57, wherein the immunization is performed with cells which have been transfected with human CD20.

59. An isolated human monoclonal antibody which binds to human CD20 obtained by:

immunizing a transgenic non-human animal having a genome comprising a human heavy chain transgene and a human light chain transgene with a cell which has been transfected with human CD20, such that antibodies are produced by B cells of the animal;

isolating B cells of the animal;

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fusing the B cells with myeloma cells to form immortal, hybridoma cells that secrete human monoclonal antibodies specific for human CD20; and

isolating the human monoclonal antibodies specific for CD20 from the culture supernatant of the hybridoma, or the transfectoma derived from such hybridoma.

60. An isolated human antibody comprising a heavy chain variable region amino acid sequence derived from a human V_H3-13/DP-44 germline sequence (SEQ ID NO:54) and a light chain variable region amino acid sequence derived from a human L6/JK4-CK (SEQ ID NO:55) germline sequence, wherein the human antibody binds to human CD20.

61. An isolated human antibody comprising a heavy chain variable region amino acid sequence derived from a human V_H3-09/JH6b germline sequence (SEQ ID NO:56) and a light chain variable region amino acid sequence derived from a human V_L-L6/JK5 germline sequence (SEQ ID NO:57), wherein the human antibody binds to human CD20.

62. A composition comprising the human antibody of any one of claims 1-45 and a pharmaceutically acceptable carrier.

63. A composition comprising a combination of two or more human antibodies according to any one of claims 1-45, which have complementary functional activities.

64. A composition according to claim 61 comprising a first antibody according to claim 15 and a second antibody according to claim 19.

65. A composition according to any of claims 62-64 further comprising a therapeutic agent.

66. The antibody according to any one of claims 1-45, further comprising a chelator linker for attaching a radioisotope.

67. An immunoconjugate comprising an antibody according to any one of claims 1-45 linked to a cytotoxic agent, a radioisotope, or a drug.

68. A bispecific molecule comprising an antibody according to any one of claims 1-45 and a binding specificity for a human effector cell.

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69. A bispecific molecule comprising an antibody according to any one of claims 1-45 and a binding specificity for a human Fc receptor or a binding specificity for a T cell receptor, such as CD3.

70. A method of inhibiting growth of a cell expressing CD20, comprising contacting the cell with an effective amount of an antibody according to any one of claims 1-45 such that the growth of the cell is inhibited.

71. A method of killing a cell expressing CD20, comprising contacting the cell with the antibody of any one of claims 1-45, such that killing of the cell expressing CD20 occurs.

72. The method of any one of claims 70 or 71, wherein the cell is a B lymphocyte or a tumor cell.

73. A method of treating or preventing a disease or disorder involving cells expressing CD20, comprising administering to a subject a human antibody, composition, immunoconjugate, or bispecific molecule according to any one of claims 1-45 or 60-69, an expression vector according to any one of the claims 94-97, in an amount effective to treat or prevent the disease.

74. The method of claim 73, wherein the disease is a B cell lymphoma.

75. The method of claim 73, wherein the disease is B cell non-Hodgkin's lymphoma.

76. The method of claim 73, wherein the disease is selected from the group consisting of precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, such as B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma (MALT type, nodal and splenic type), hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenström's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL).

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77. The method of claim 76, wherein the disease is follicular lymphoma (FL).

78. The method of claim 76, wherein the disease is B cell chronic lymhocytic leukemia (CLL)/small lymphocytic lymphoma (SLL).

79. The method of claim 73, wherein the disease is selected from the group consisting of lymphomatoid granulomatosis, primary effusion lymphoma, intravascular large B cell lymphoma, mediastinal large B cell lymphoma, heavy chain diseases (including γ, μ, and α disease), lymphomas induced by therapy with immunosuppressive agents, such as cyclosporine-induced lymphoma, and methotrexate-induced lymphoma.

80. A method of treating or preventing an immune disease involving CD20 expressing immune cells, comprising administering to a subject the antibody, composition, immunoconjugate, or bispecific molecule according to any one of claims 1-45 or 60-67 or an expression vector according to any one of the claims 94-97, in an amount effective to treat or prevent the immune disease.

81. The method of claim 80, wherein treatment includes the killing of B cells which produce antibodies against autoantigens.

82. The method of claim 73, wherein the disease or disorder is selected from the group consisting of psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behçet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis (RA), atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases.

83. The method of claim 82, wherein the autoimmune disease is rheumatoid arthritis (RA).

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84. The method of claim 73, wherein the disease is an inflammatory, immune and/or autoimmune disorder selected from ulcerative colitis, Crohn's disease, juvenile onset diabetes, multiple sclerosis, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, systemic sclerosis, and pemphigus vulgaris.

85. The method of claim 73, wherein the disease is an inflammatory, immune and/or autoimmune disorder selected from inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, and multiple sclerosis.

86. The method of any one of claims 70-85, further comprising separately administering another therapeutic agent to the subject.

87. The method of claim 86, wherein the therapeutic agent is a cytotoxic agent or a radiotoxic agent.

88. The method of claim 86, wherein the therapeutic agent is an immunosuppressant.

89. The method of claim 86, wherein the therapeutic agent is an immunological modulating agent, such as a cytokine or a chemokine.

90. The method of claim 86, wherein the therapeutic agent is selected from the group consisting of doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide.

91. The method of claim 86, wherein the therapeutic agent is selected from the group consisting of anti-CD25 antibodies, anti-CD19 antibodies, anti-CD21 antibodies, anti-CD22 antibodies, anti-CD37 antibodies, anti-CD38 antibodies, anti-IL6R antibodies, anti-IL8 antibodies, anti-IL15 antibodies, anti-IL15R antibodies, anti-CD4 antibodies, anti-CD11a antibodies, anti-alpha-4/beta-1 integrin (VLA4) antibodies, CTLA4-Ig, and anti-C3b(i) antibodies.

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92. An *in vitro* method for detecting the presence of CD20 antigen, or a cell expressing CD20, in a sample comprising:

contacting the sample with the antibody of any one of claims 1-45 under conditions that allow for formation of a complex between the antibody and CD20; and

detecting the formation of a complex.

93. A kit for detecting the presence of CD20 antigen , or a cell expressing CD20, in a sample comprising the antibody of any one of claims 1-45.

94. An *in vivo* method for detecting CD20 antigen, or a cell expressing CD20, in an subject comprising:

administering the antibody of any one of claims 1-45 under conditions that allow for formation of a complex between the antibody and CD20; and

detecting the formed complex.

95. An expression vector comprising a nucleotide sequence encoding the variable region of a light chain, heavy chain or both light and heavy chains of a human antibody which binds human CD20.

96. The expression vector of claim 95, further comprising a nucleotide sequence encoding the constant region of a light chain, heavy chain or both light and heavy chains of a human antibody which binds human CD20.

97. An expression vector comprising a nucleotide sequence encoding a heavy chain variable region comprising a nucleotide selected from the group consisting of the nucleotide sequences as set forth in SEQ ID NOs: 1, 5, and 9, and a light variable region comprising a nucleotide sequence selected from the group consisting of the nucleotide sequences as set forth in SEQ ID NOs: 3, 7, and 11, and conservative modifications thereof.

98. An expression vector comprising a nucleotide sequence encoding a heavy chain variable region comprising an amino acid sequence selected from the group consisting of the amino acid sequences as set forth in SEQ ID NOs:2, 6, and 10, and a light chain variable region comprising the amino acid sequence selected from the group consisting of the amino acid sequences as set forth in shown in SEQ ID NOs: 4, 8, and 12, and conservative sequence modifications thereof.

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99. A pharmaceutical composition comprising the expression vector of any one of claims 95-98 and a pharmaceutically acceptable carrier.

100. An anti-idiotypic antibody binding to an antibody of any one of claims 1-45.

101. An anti-idiotypic antibody binding to 2F2, 11B8 or 7D8.

102. Use of an anti-idiotypic antibody of claim 100 or 101 for detecting the level of human monoclonal antibody against CD20 in a sample.

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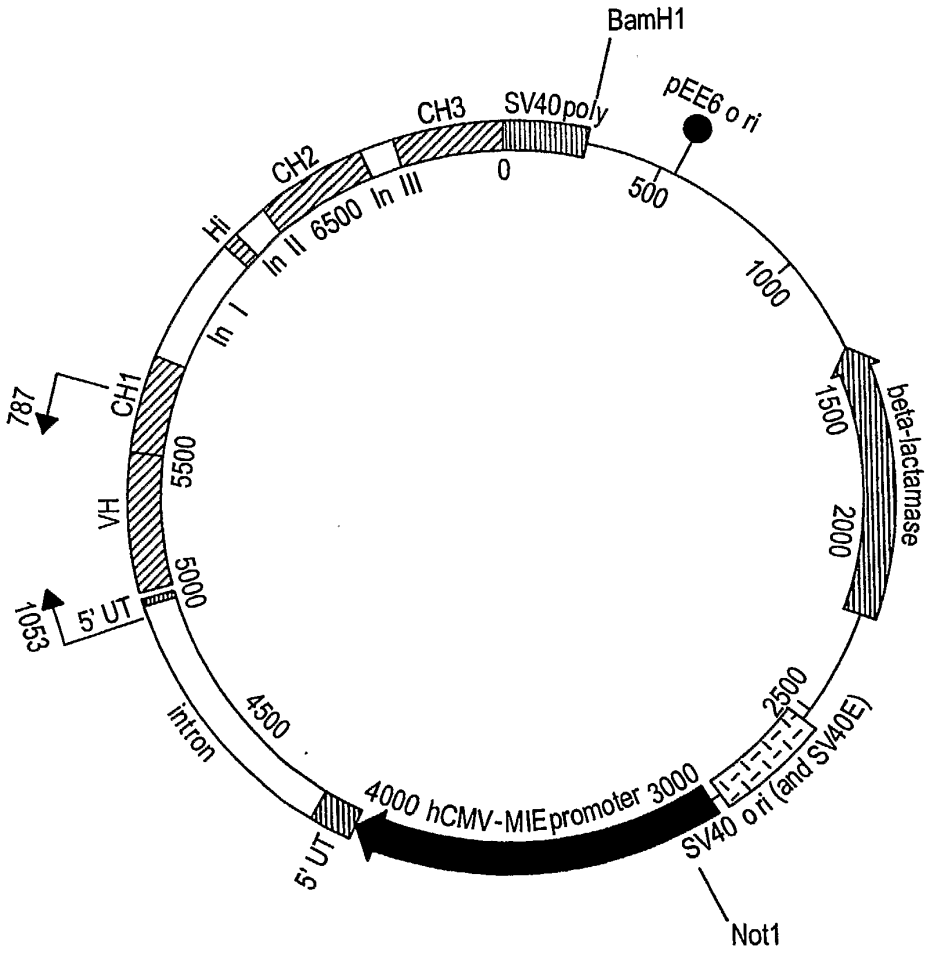

BamH1
pEE6 o ri
SV40poly
CH3
CH2
In III
In II 6500
In I
Hi
0
500
1000
1500
2000
2500
3000
4000 hCMV-MIEpromoter
4500
5000
5500
CH1
VH
787
1053
5' UT
intron
5' UT
SV40 o ri (and SV40E)
beta-lactamase
Not1

Fig. 1

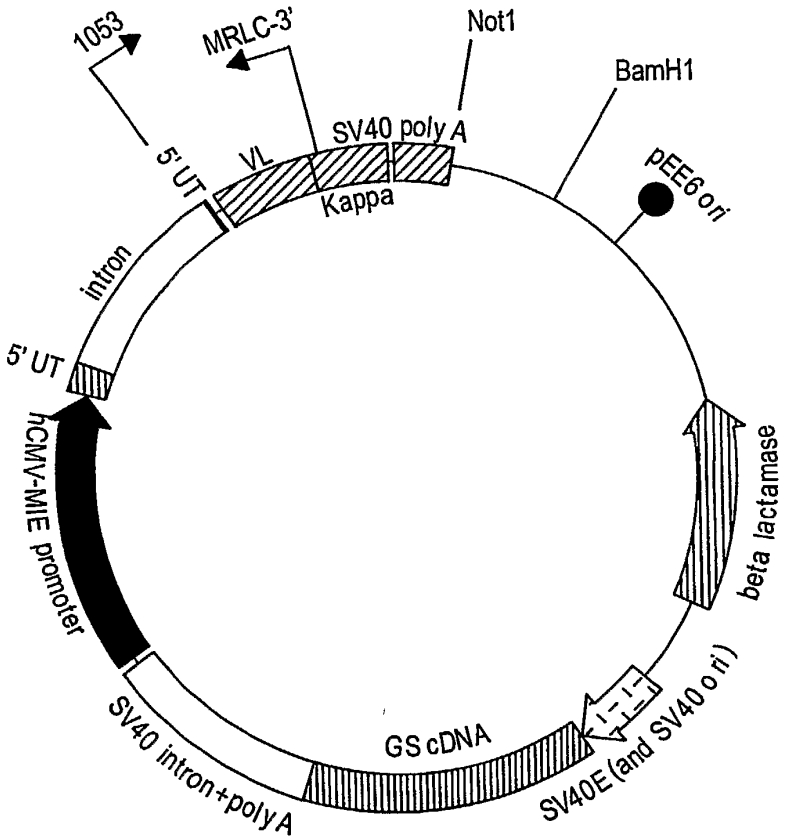


Fig. 2

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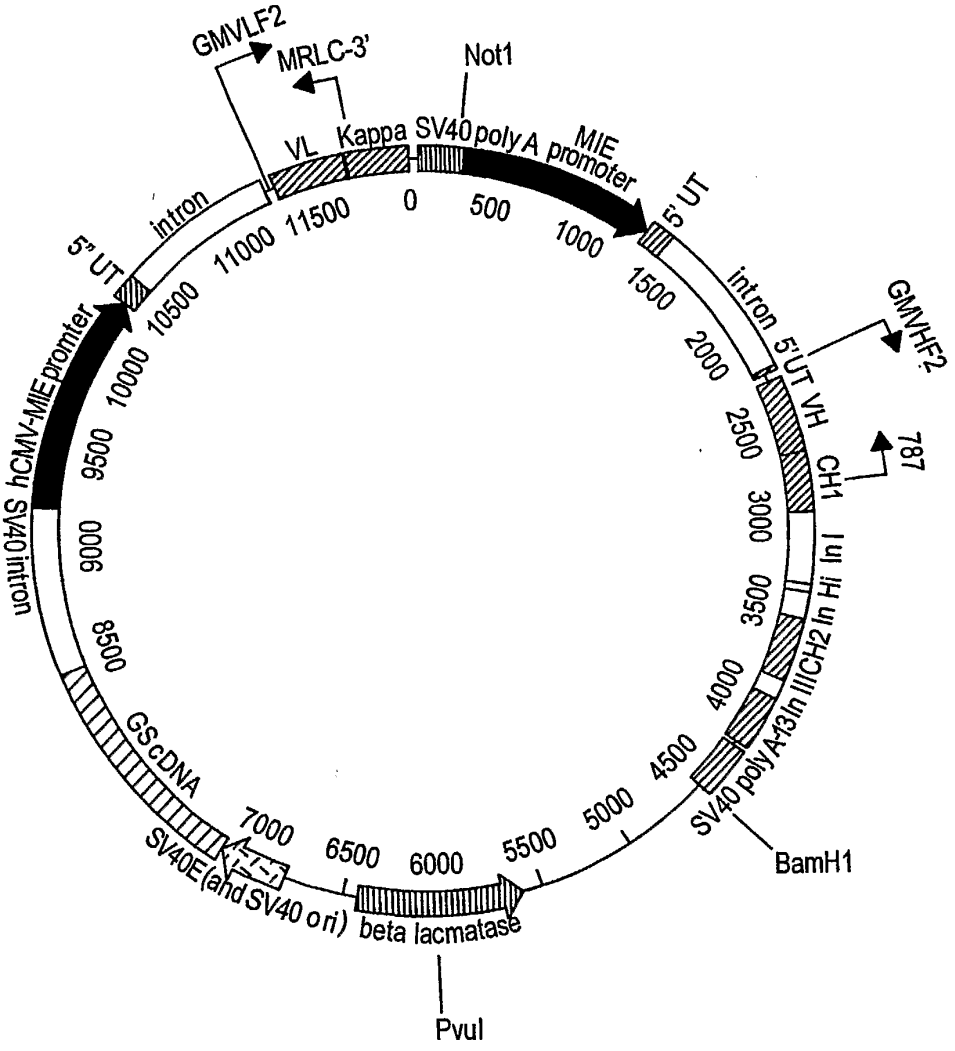


Fig. 3

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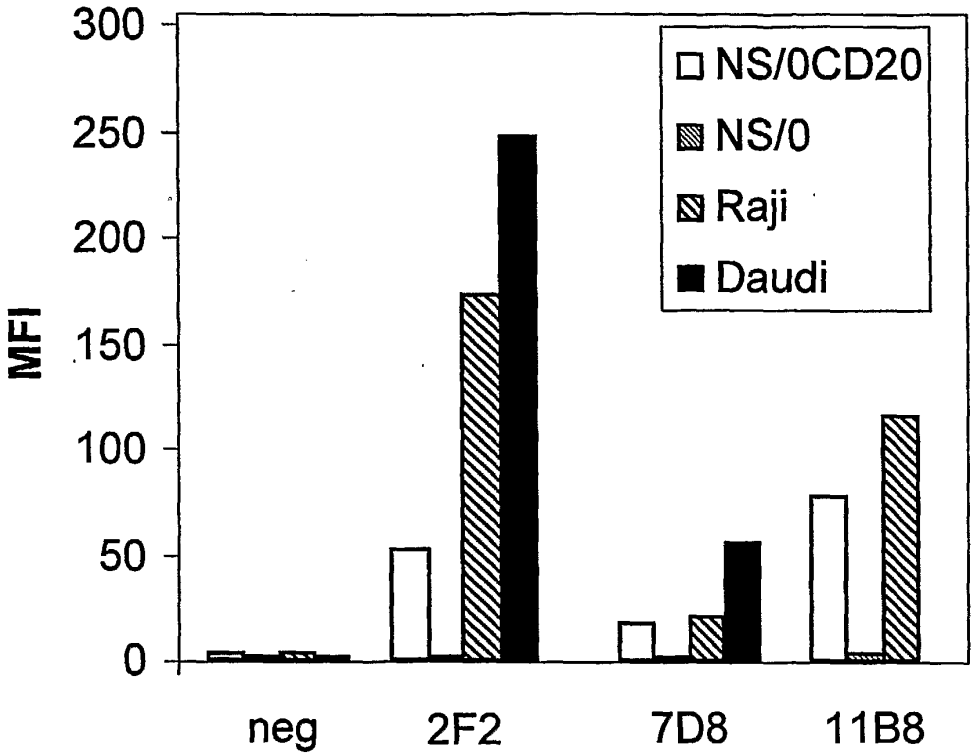

300
250
200
150
100
50
0
MFI
NS/0CD20
NS/0
Raji
Daudi
neg
2F2
7D8
11B8

Fig. 4

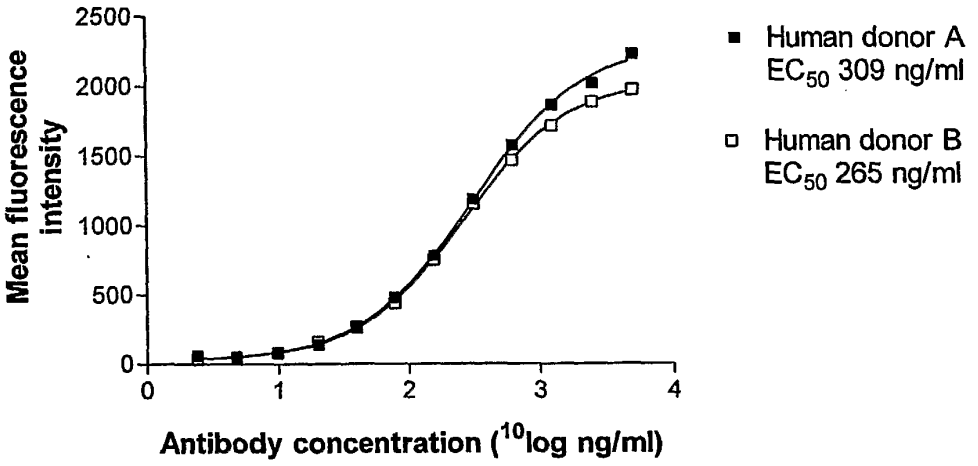

Mean fluorescence intensity
2500
2000
1500
1000
500
0
0
1
2
3
4
Antibody concentration (^{10}log ng/ml)
Human donor A
EC$_{50}$ 309 ng/ml
Human donor B
EC$_{50}$ 265 ng/ml

Fig. 5A

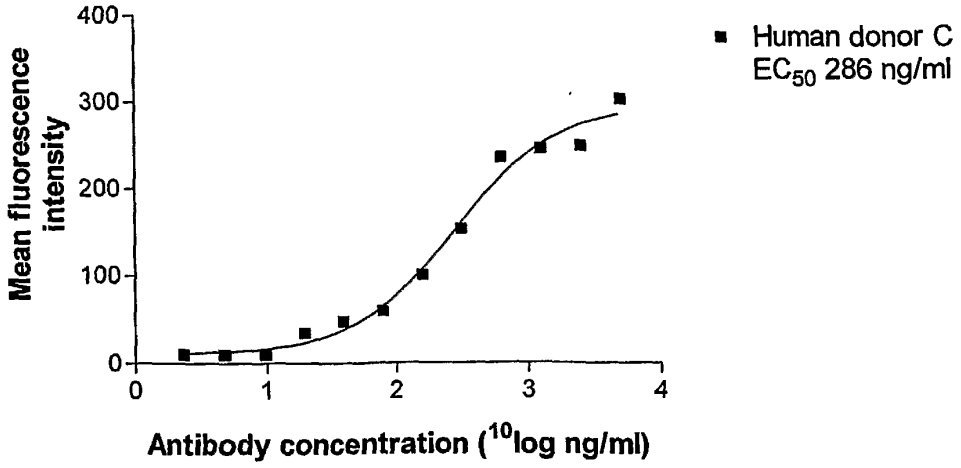

Mean fluorescence intensity
400
300
200
100
0
0
1
2
3
4
Antibody concentration (^{10}log ng/ml)
Human donor C
EC$_{50}$ 286 ng/ml

Fig. 5B

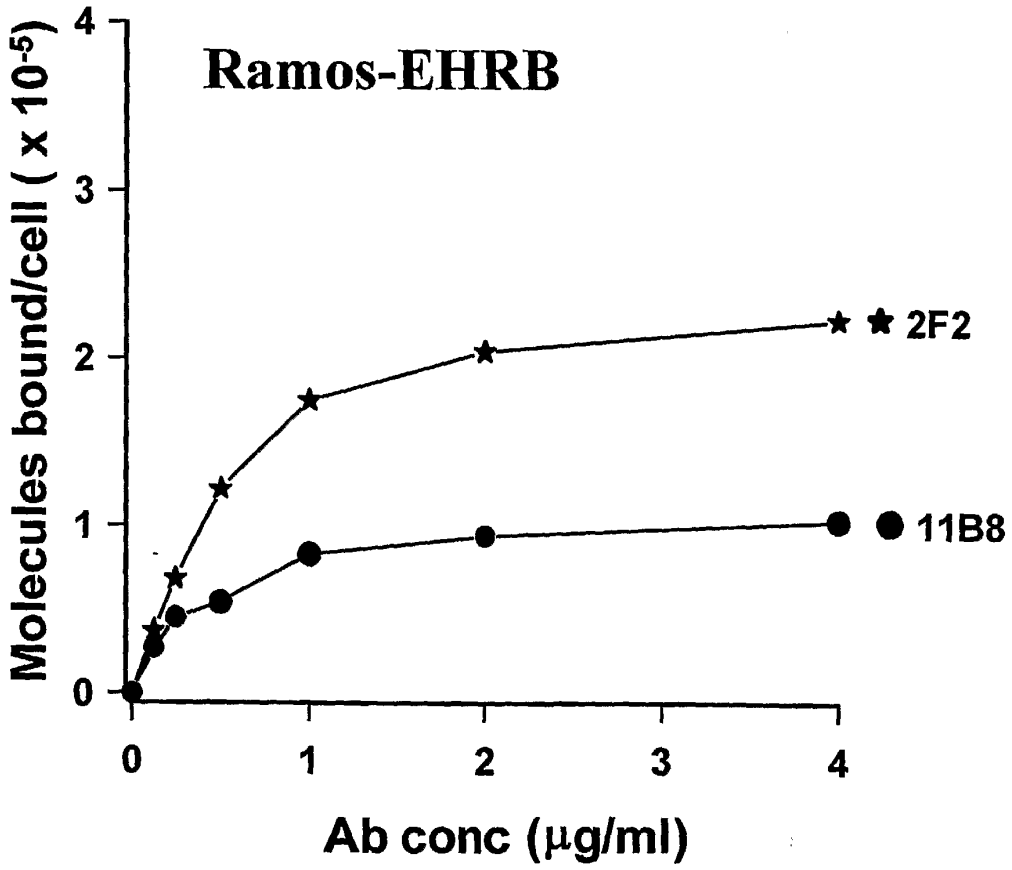

Ramos-EHRB
Molecules bound/cell (x 10-5)
4
3
2
1
0
0
1
2
3
4
Ab conc (μg/ml)
2F2
11B8

Fig 6

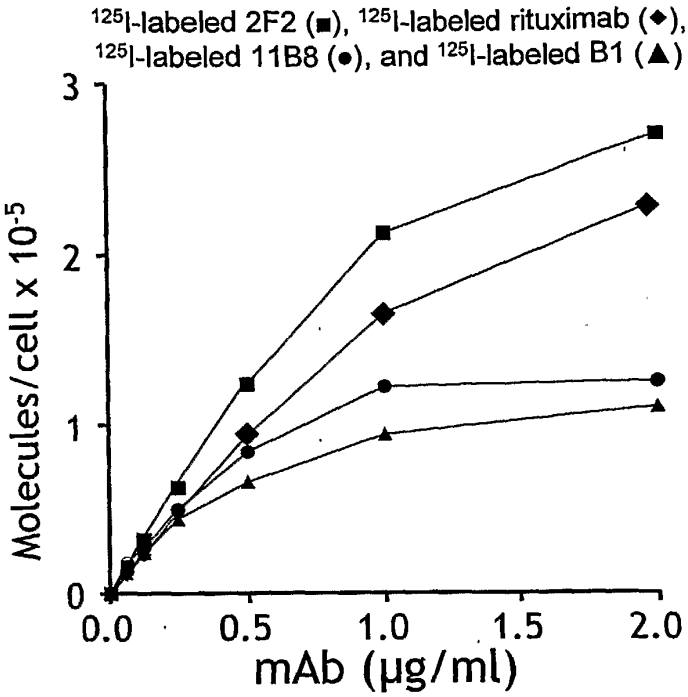

^{125}I-labeled 2F2 (■), ^{125}I-labeled rituximab (◆),
^{125}I-labeled 11B8 (●), and ^{125}I-labeled B1 (▲)
Molecules/cell x 10^{-5}
0
1
2
3
0.0
0.5
1.0
1.5
2.0
mAb (µg/ml)

Fig. 7A

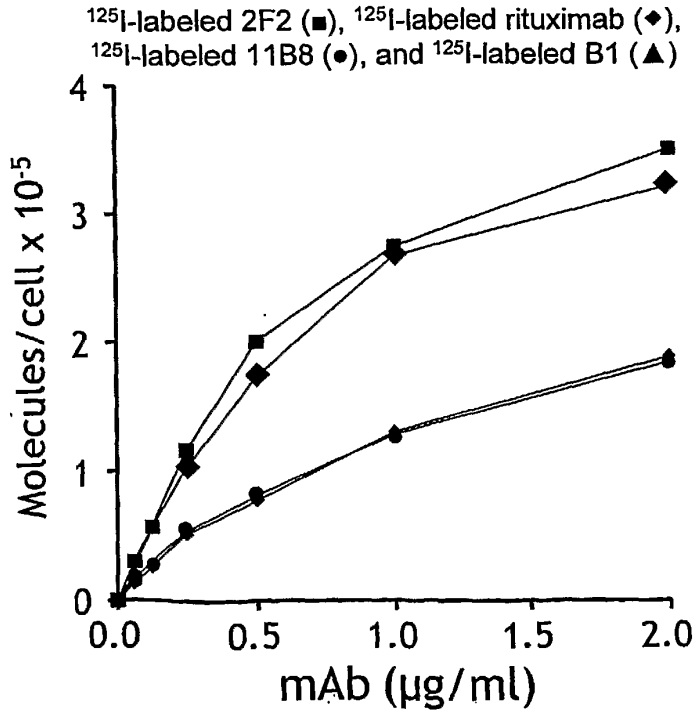

^{125}I-labeled 2F2 (■), ^{125}I-labeled rituximab (◆),
^{125}I-labeled 11B8 (●), and ^{125}I-labeled B1 (▲)
Molecules/cell x 10^{-5}
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
mAb (µg/ml)

Fig. 7B

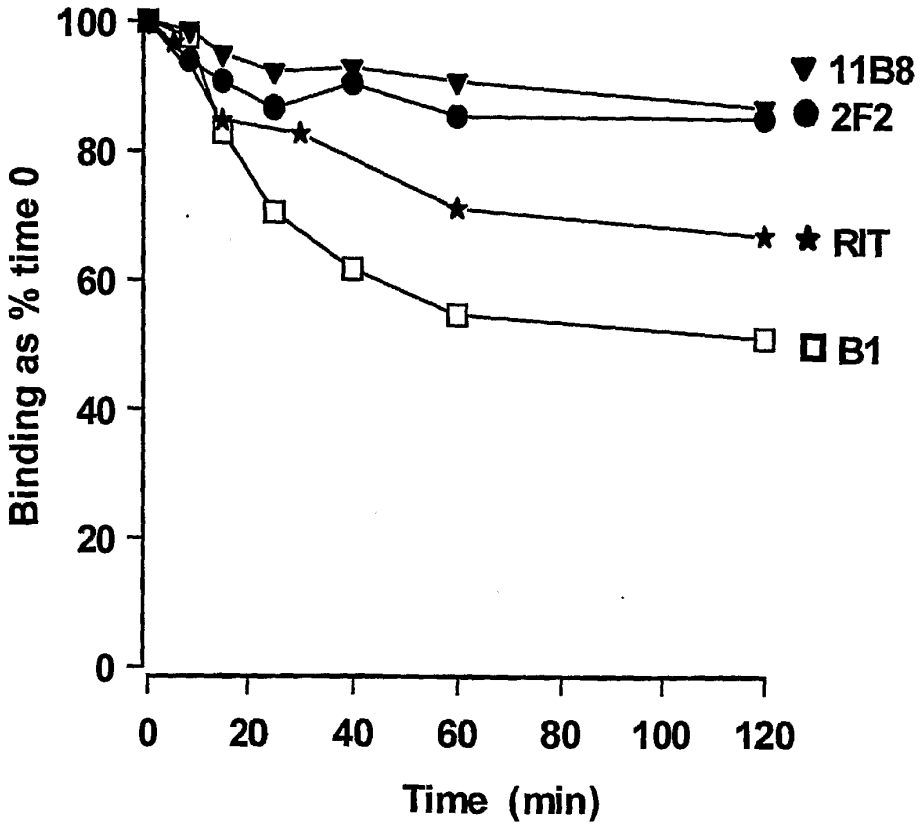


Fig. 8

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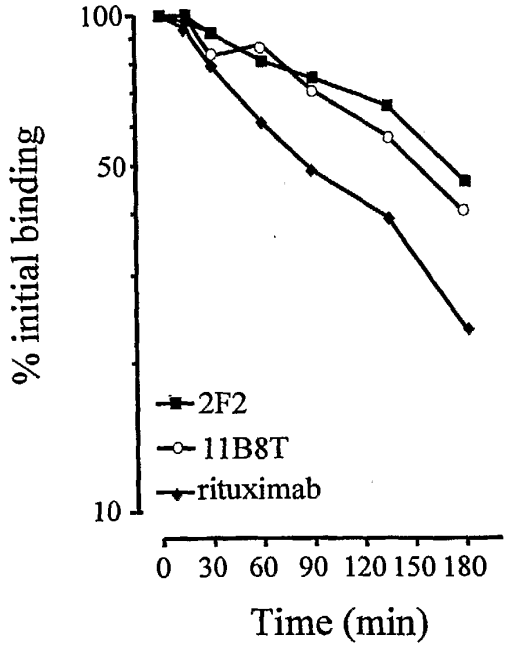


Fig. 9

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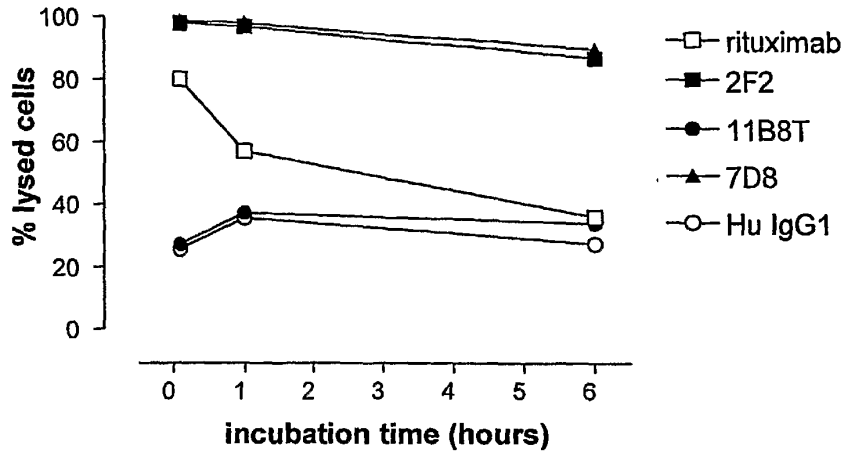


Fig. 10A

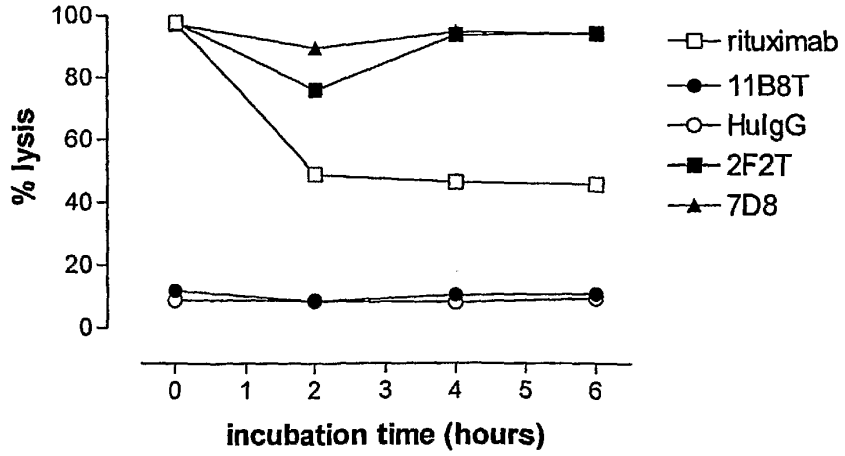


Fig. 10B

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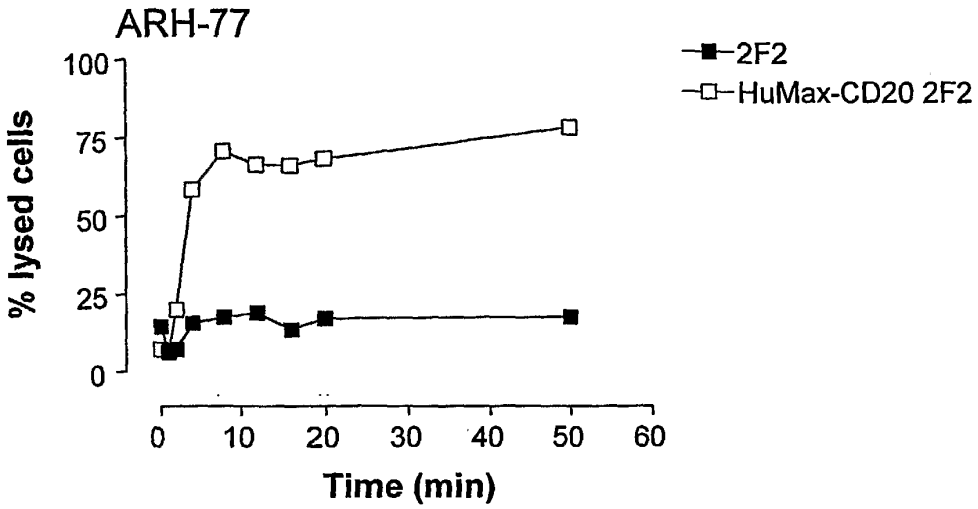


Fig. 11A

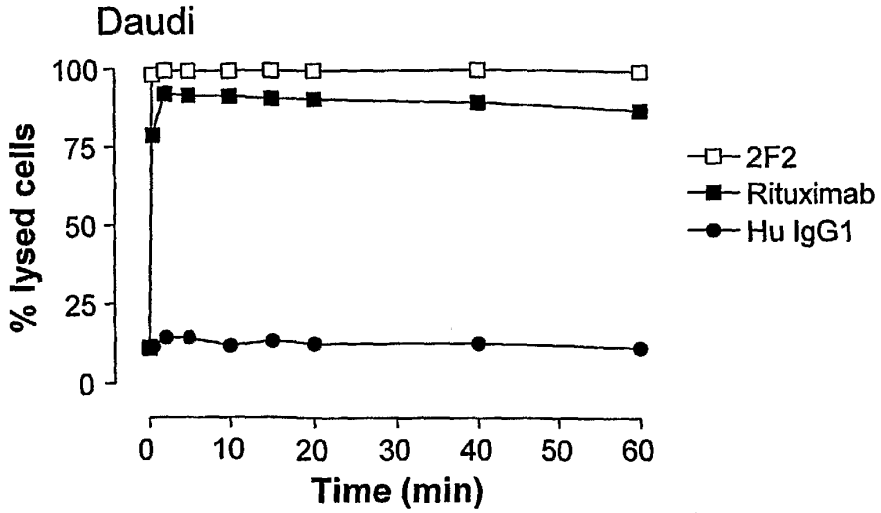


Fig. 11A

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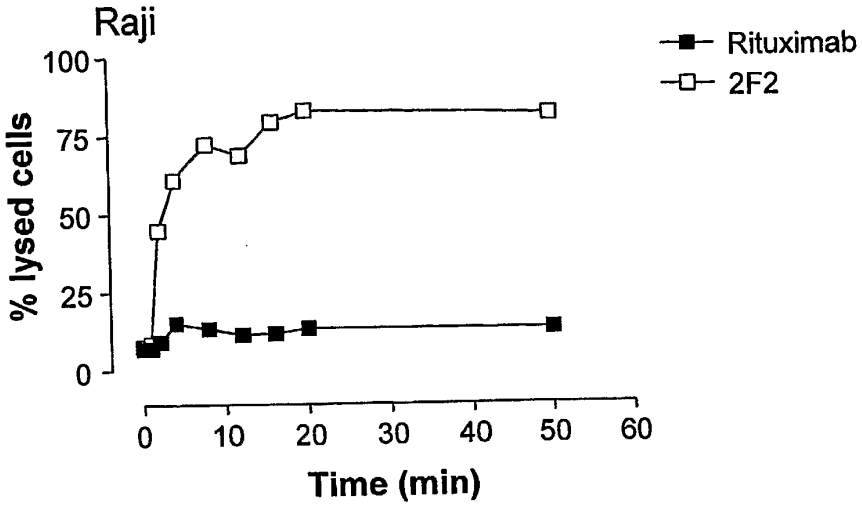


Fig. 11C

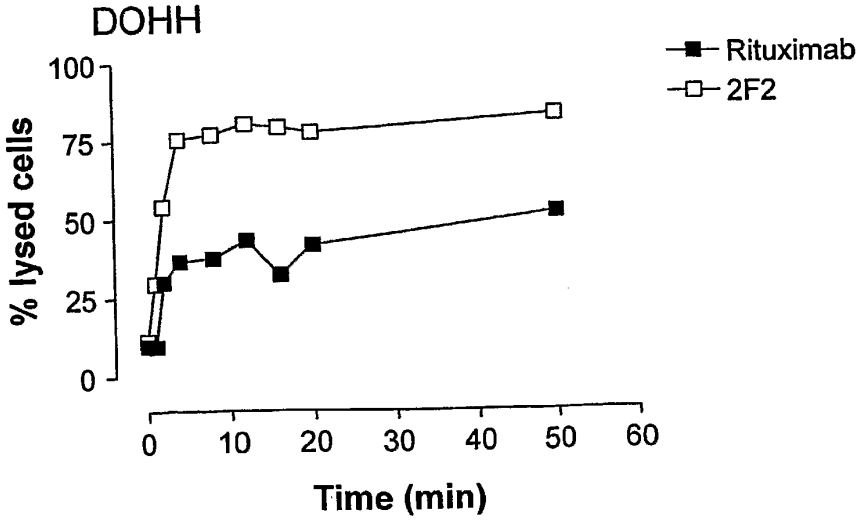


Fig. 11D

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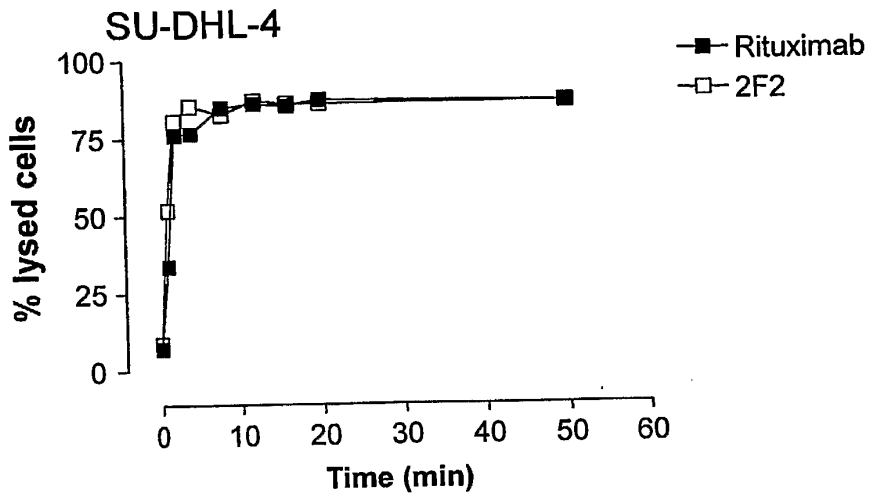


Fig. 11E

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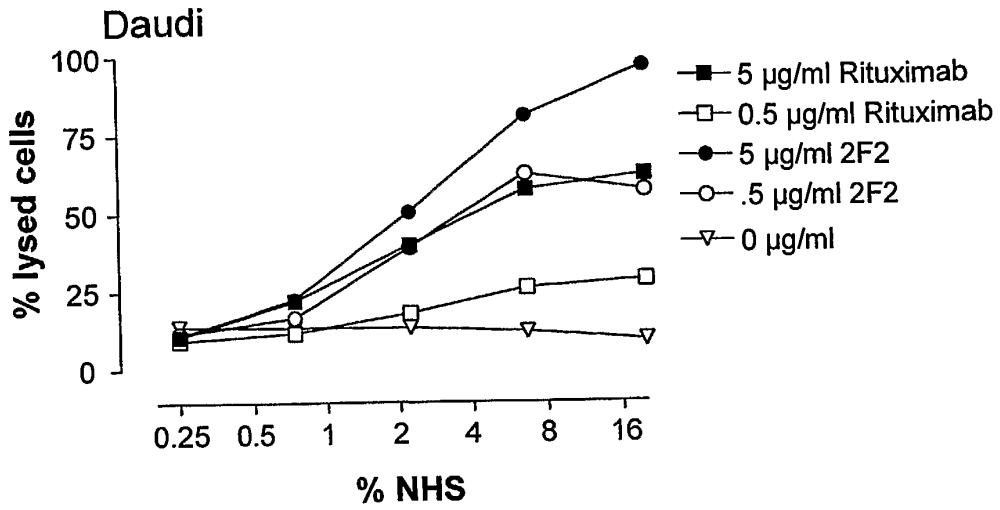


Fig. 12A

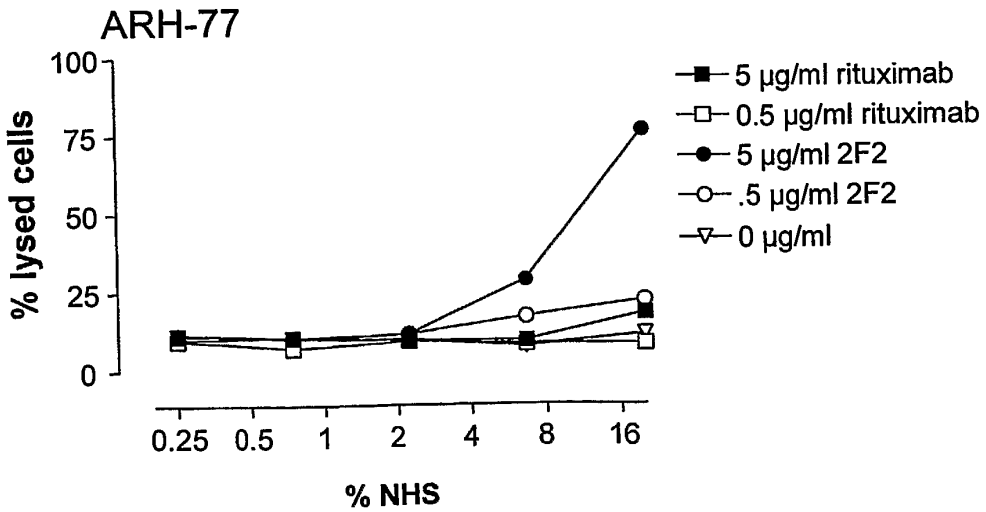


Fig. 12B

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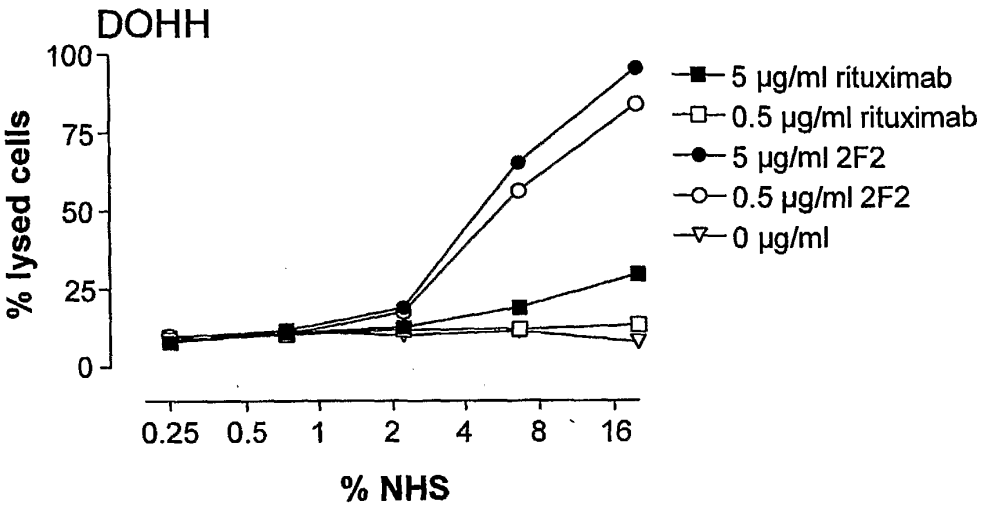


Fig. 12C

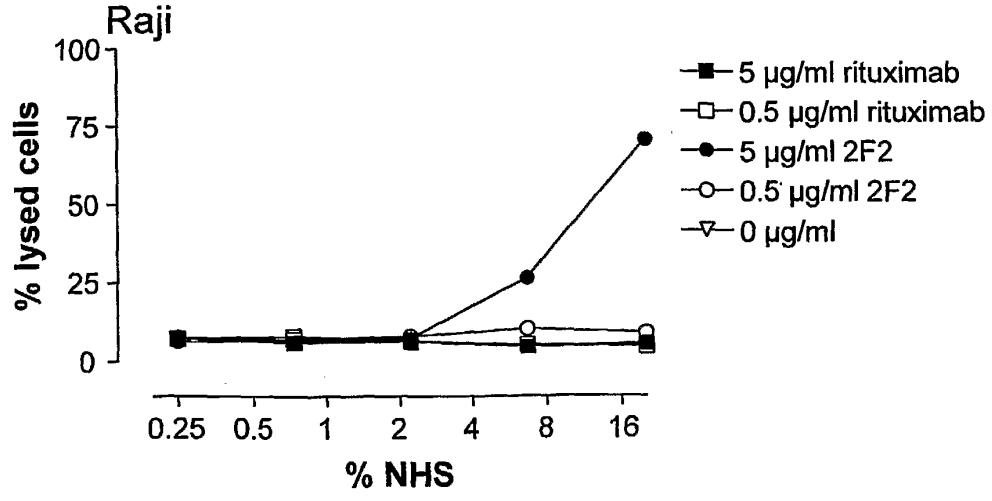


Fig. 12D

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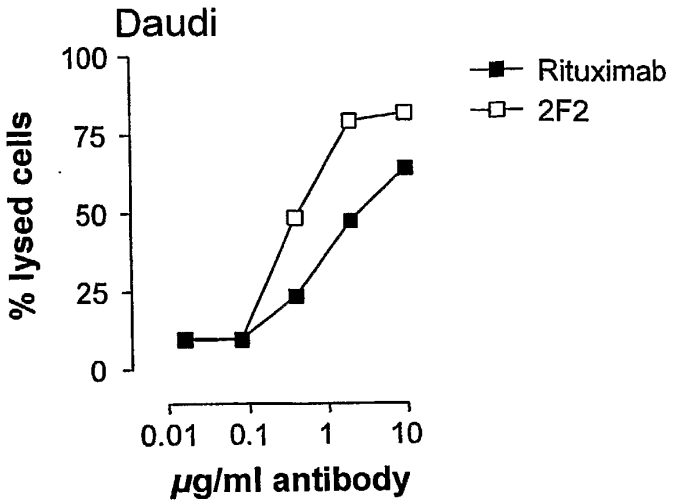


Fig. 13A

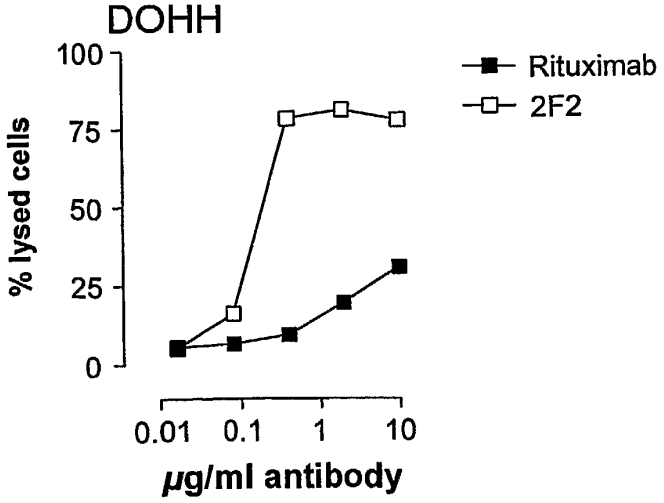


Fig. 13B

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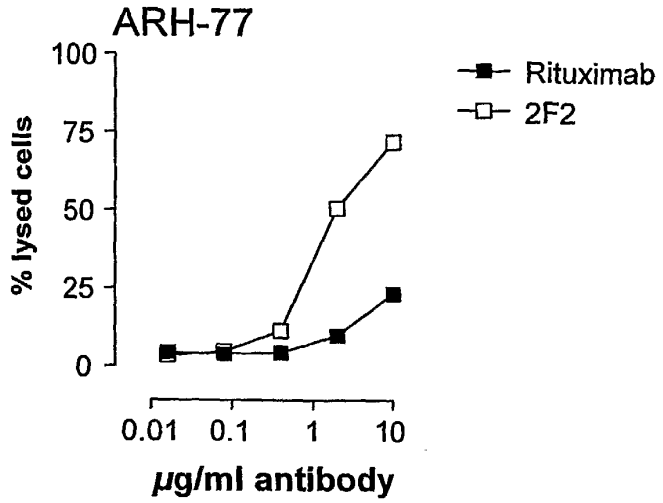


Fig. 13C

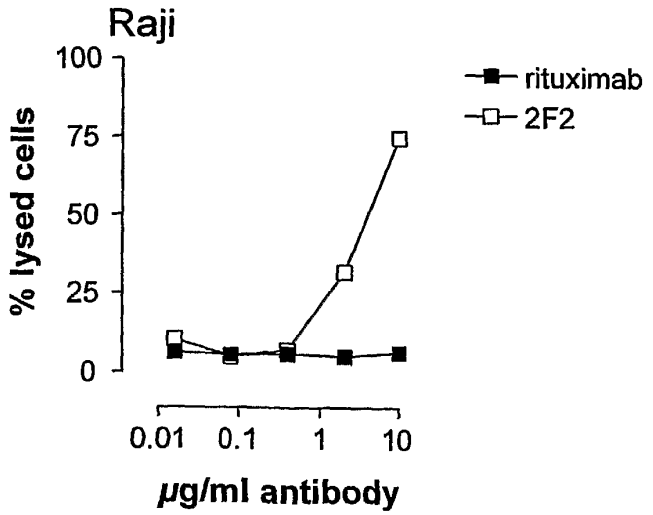


Fig. 13D

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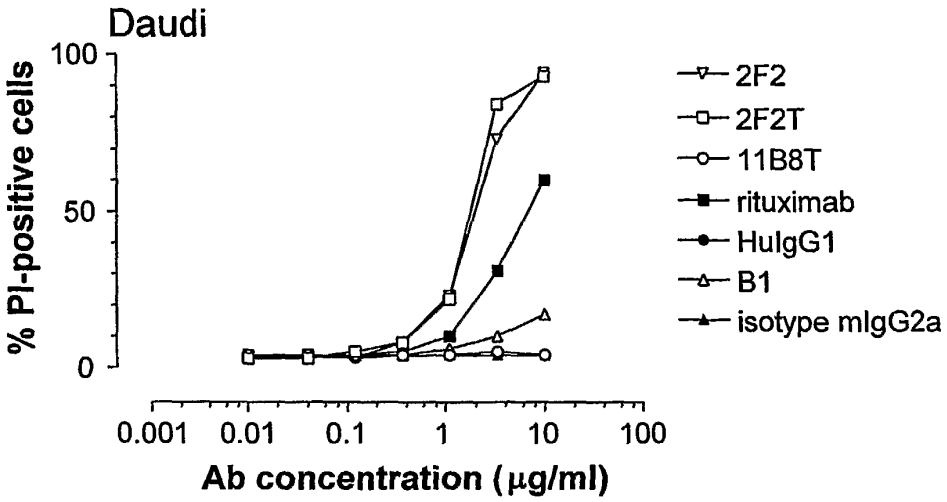


Fig. 14A

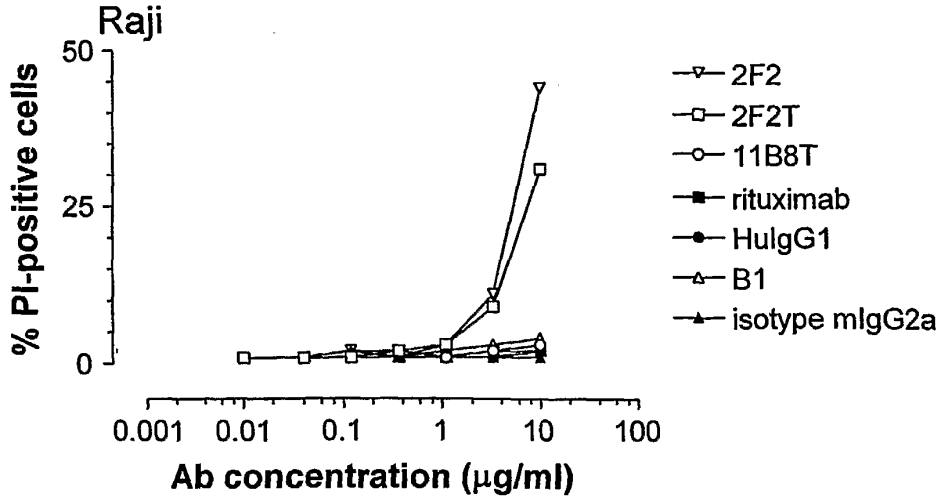


Fig. 14B

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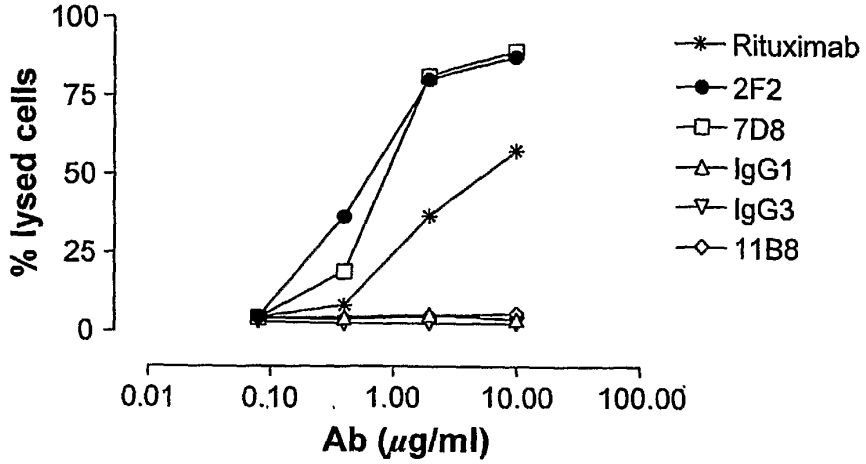


Fig. 15A

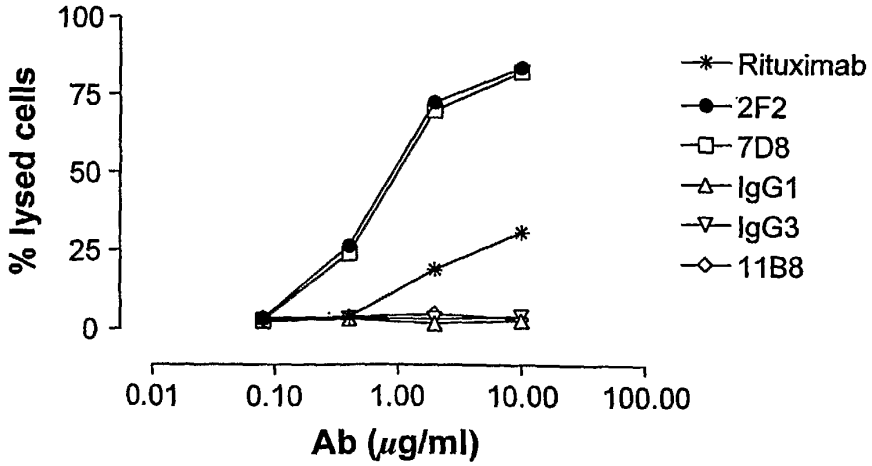


Fig. 15B

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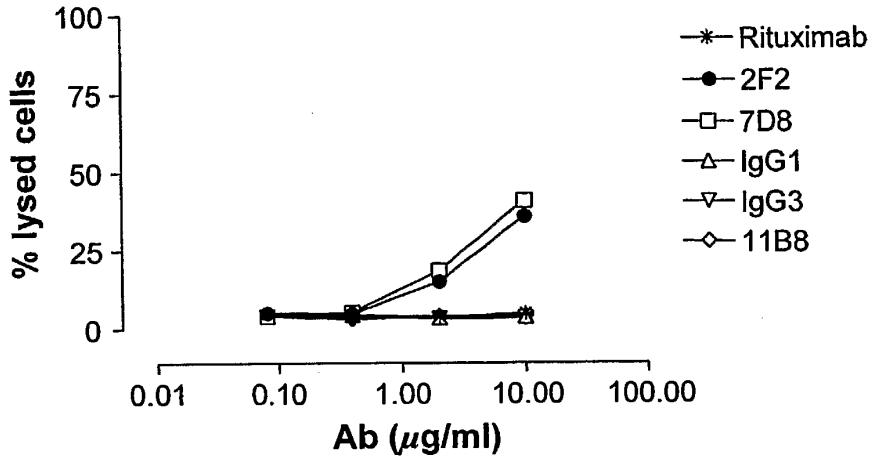


Fig. 16A

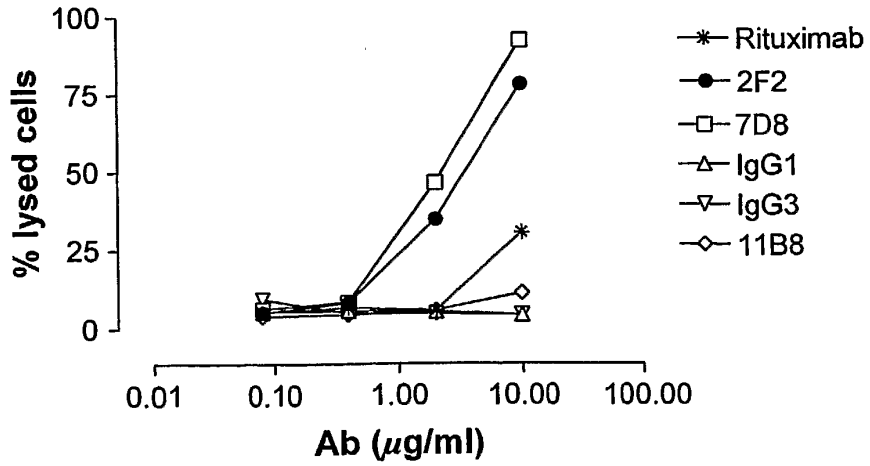


Fig. 16B

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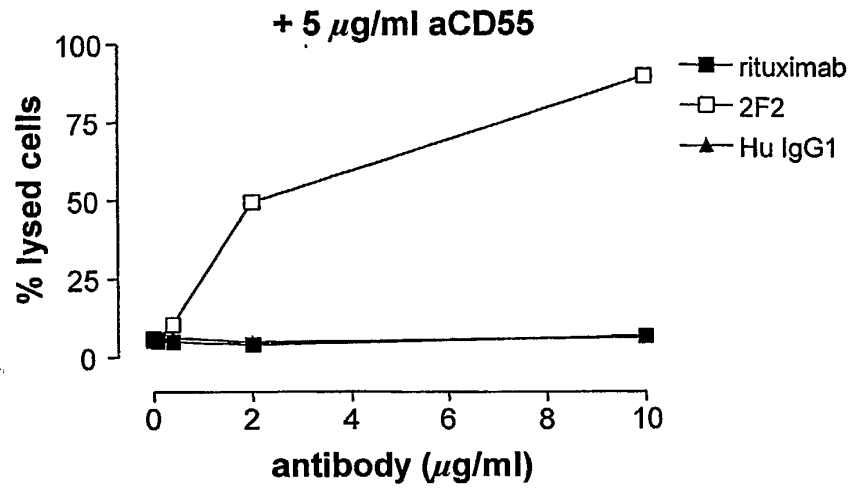


Fig. 17A

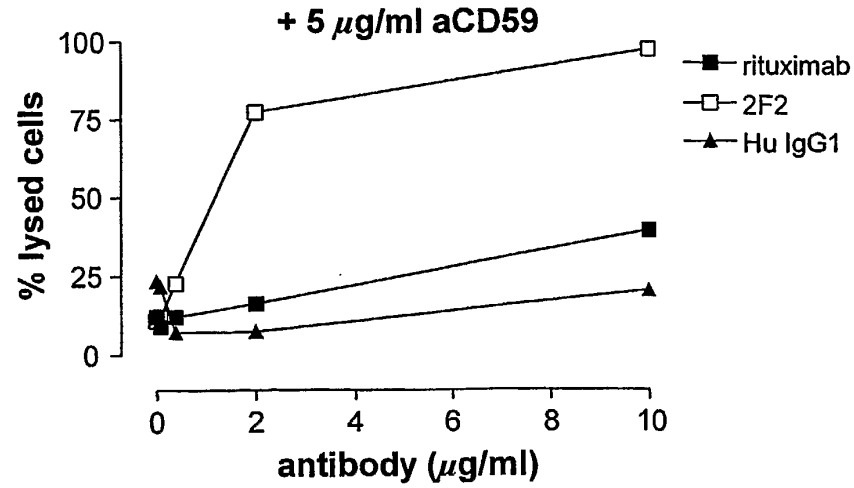


Fig. 17B

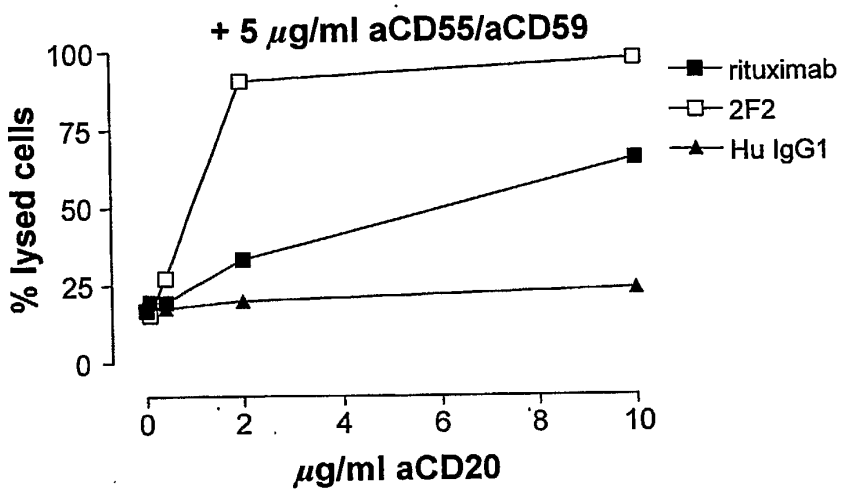


Fig. 17C

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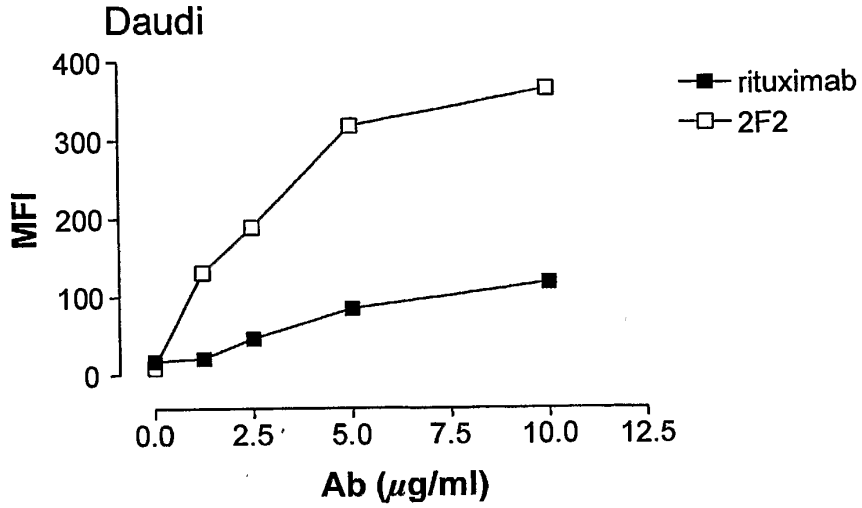


Fig. 18A

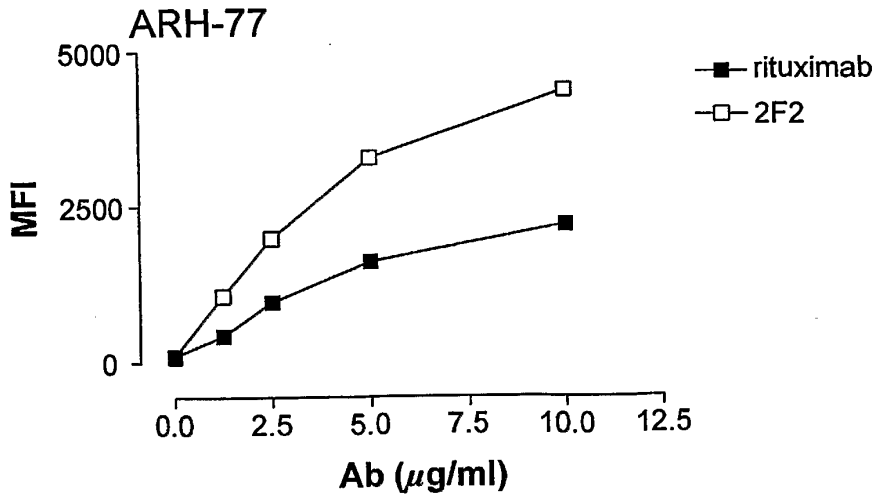


Fig. 18B

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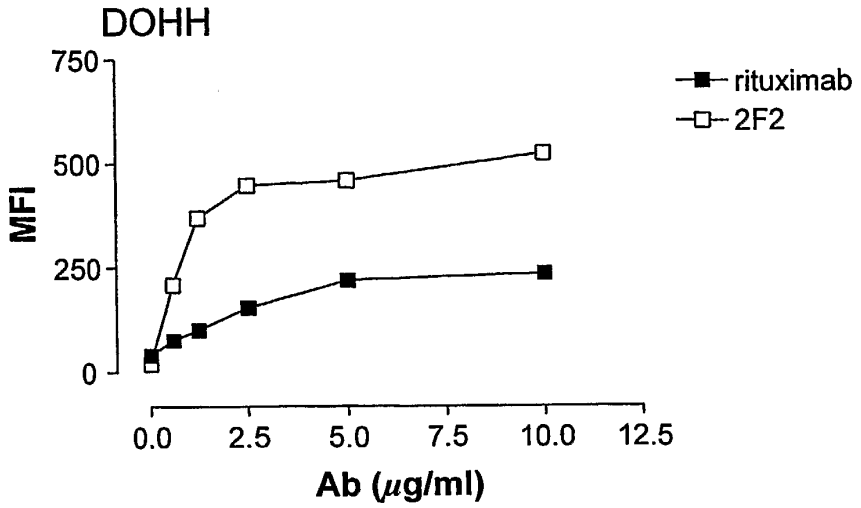


Fig. 18C

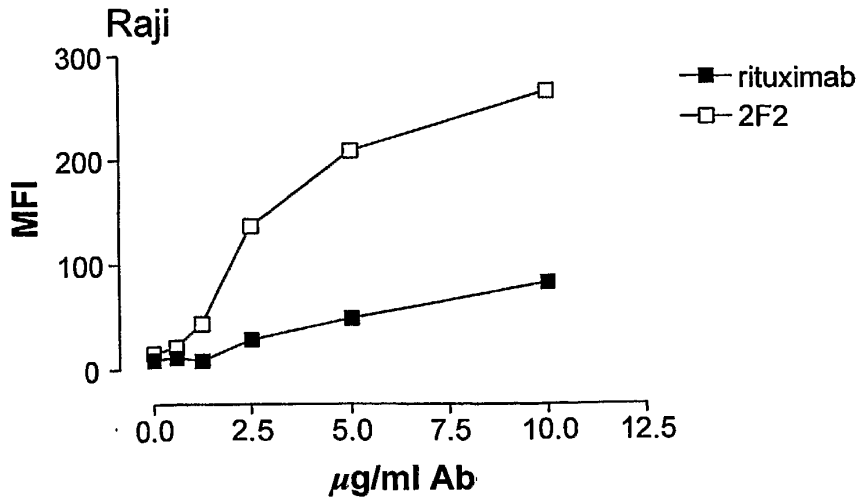


Fig. 18D

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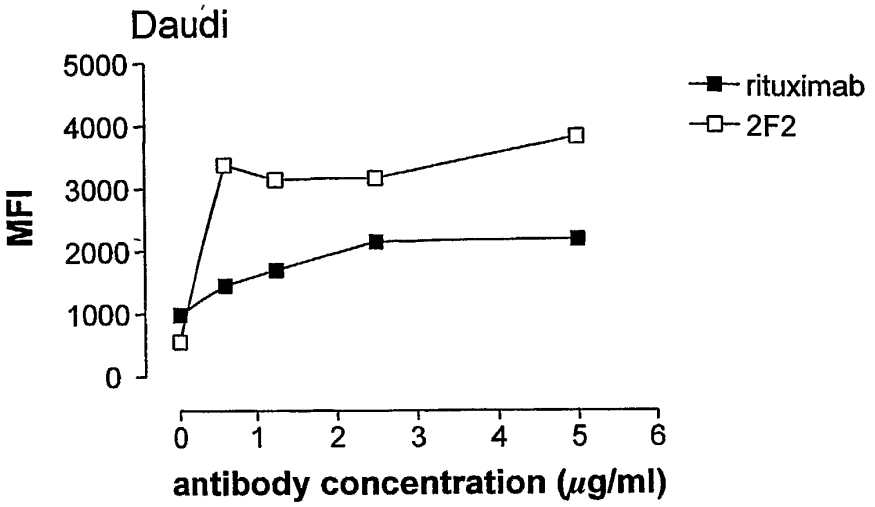


Fig. 19A

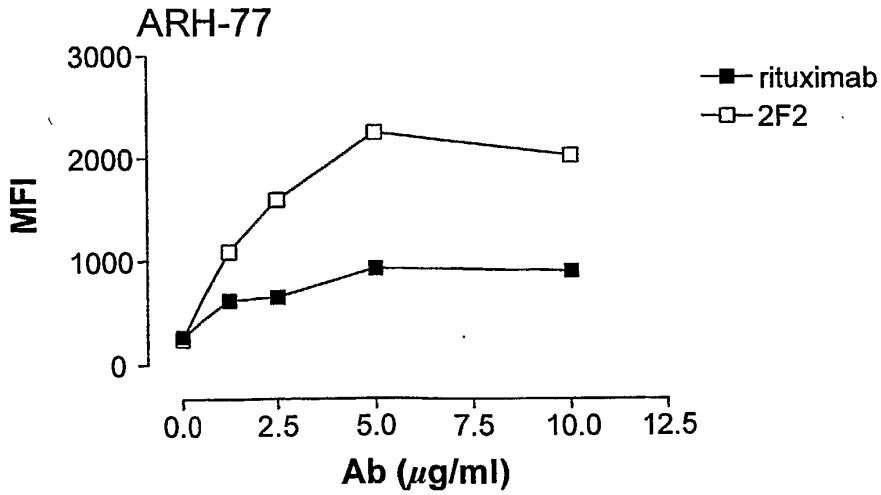


Fig. 19B

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Page 1169

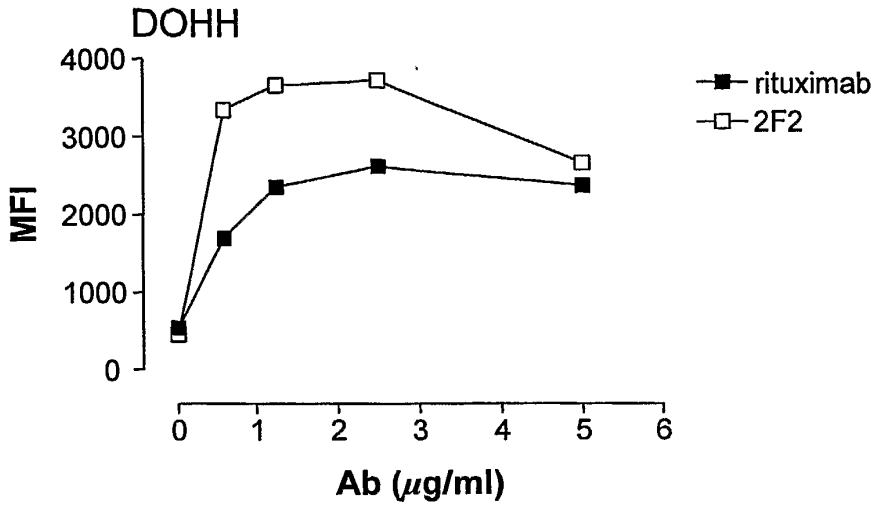


Fig. 19C

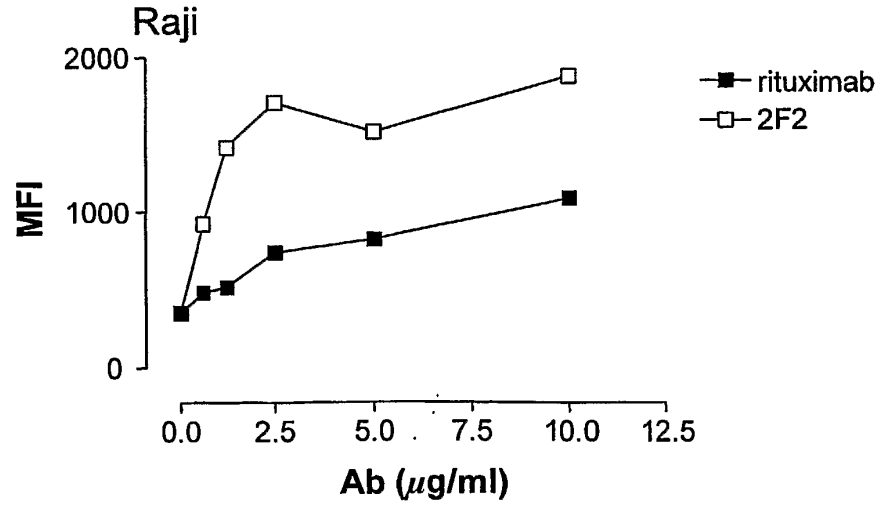


Fig. 19D

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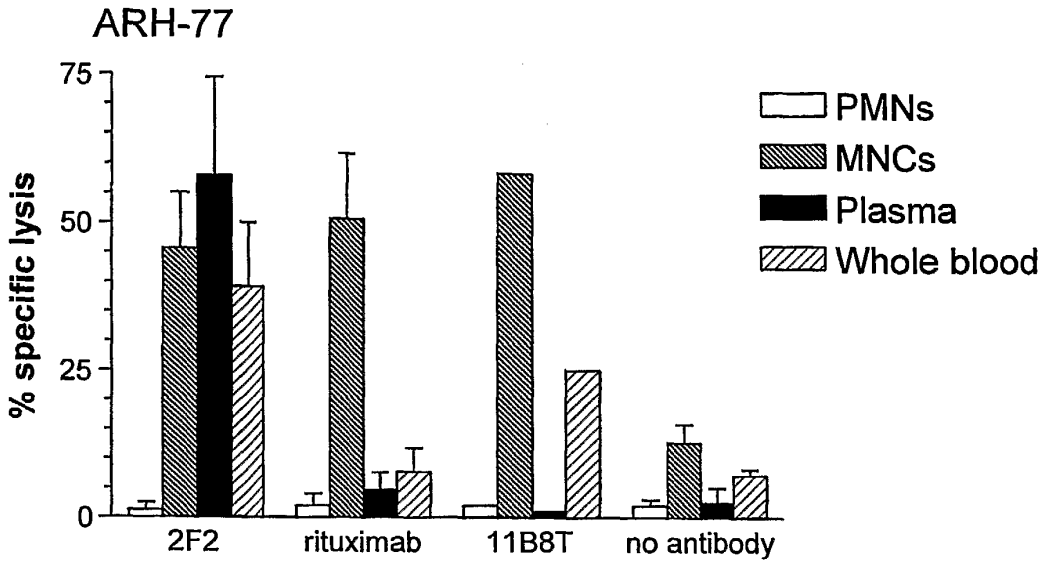


Fig. 20

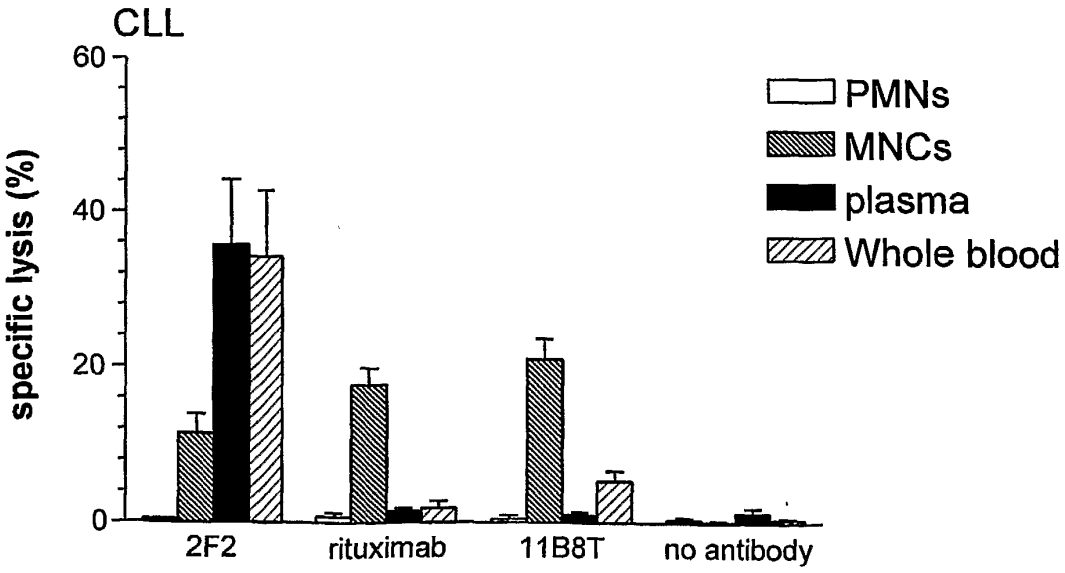


Fig. 21

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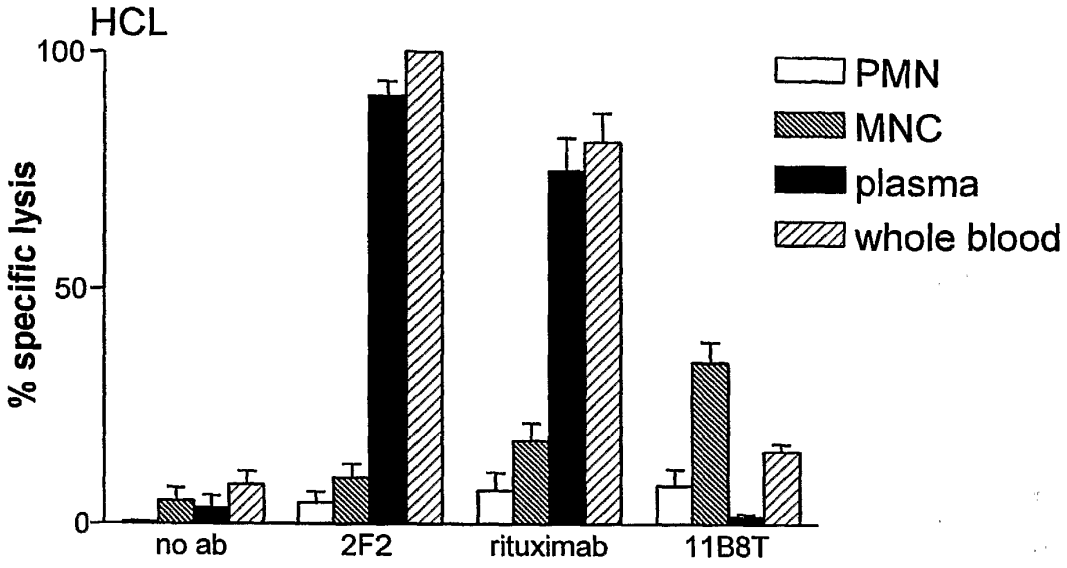

HCL
100
50
0
% specific lysis
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab
11B8T

Fig. 22

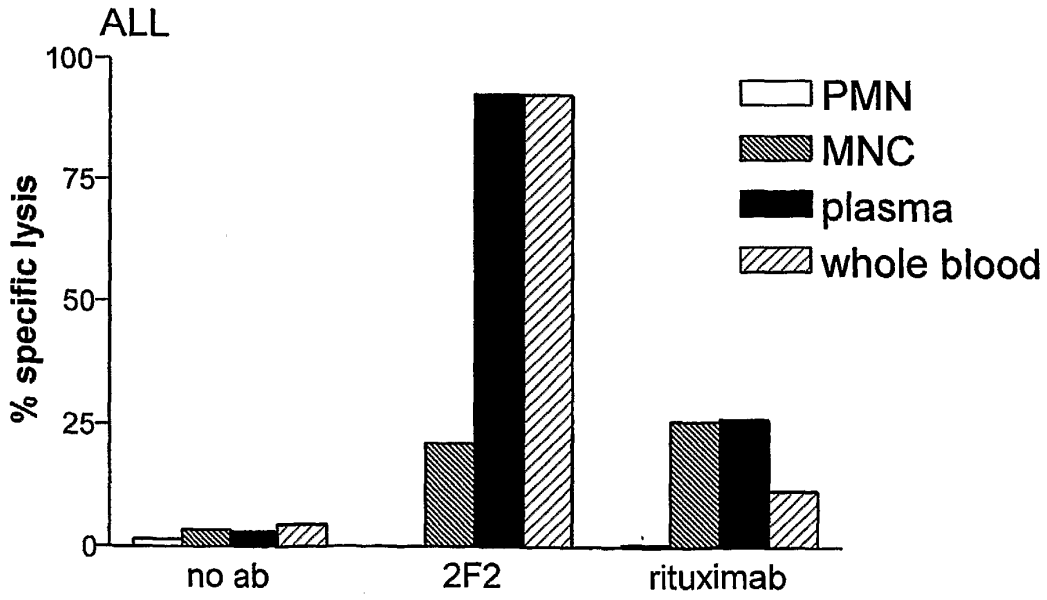

ALL
100
75
50
25
0
% specific lysis
PMN
MNC
plasma
whole blood
no ab
2F2
rituximab

Fig. 23

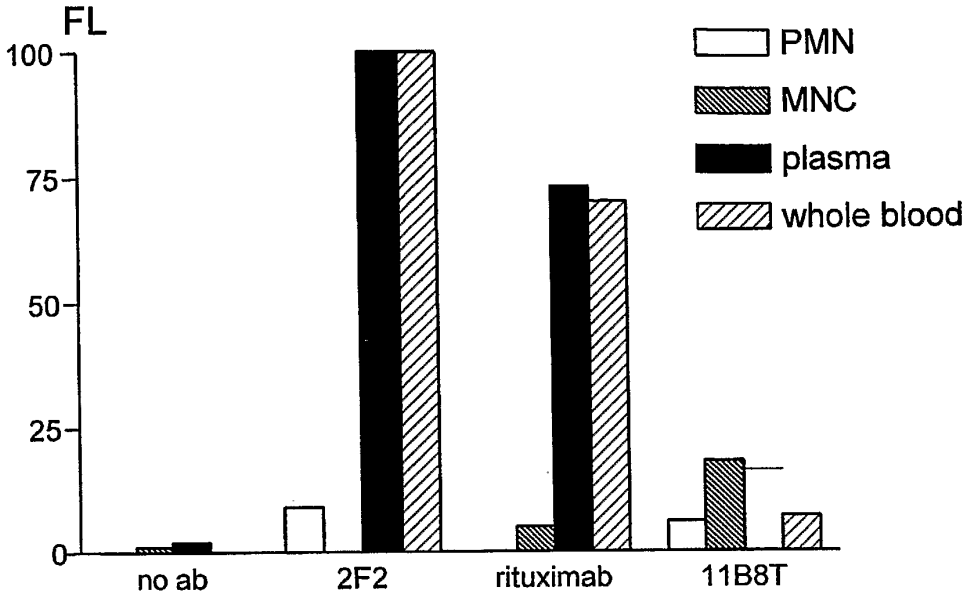


Fig. 24

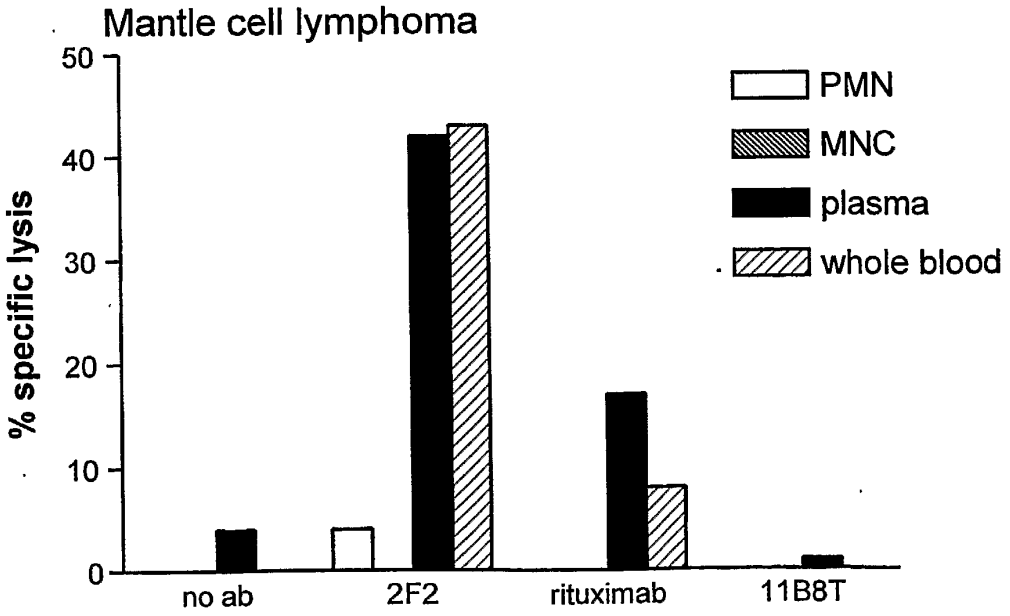


Fig. 25

Samsung Bioepis Exhibit No. 1028
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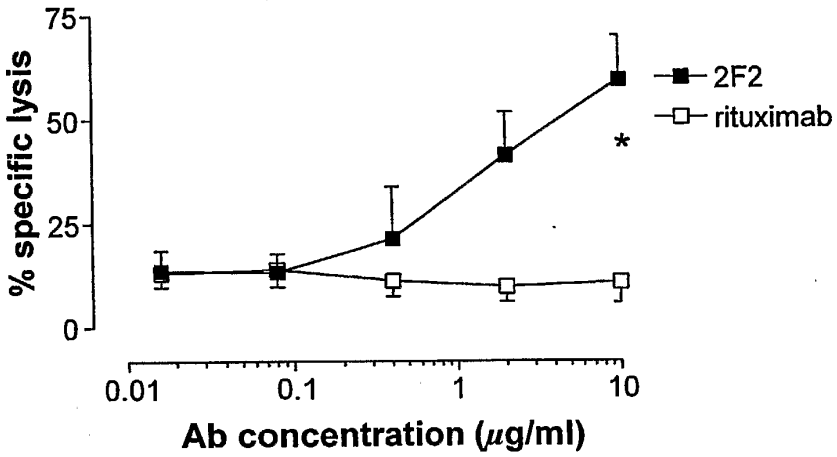


Fig. 26

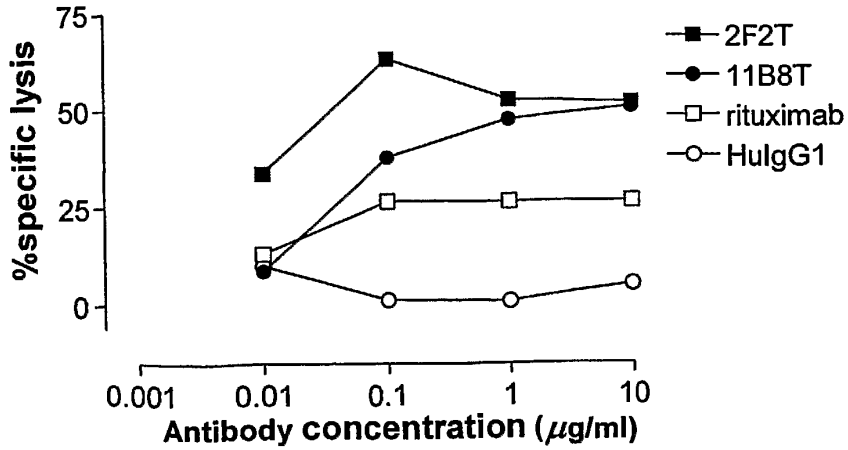


Fig. 27

Samsung Bioepis Exhibit No. 1028
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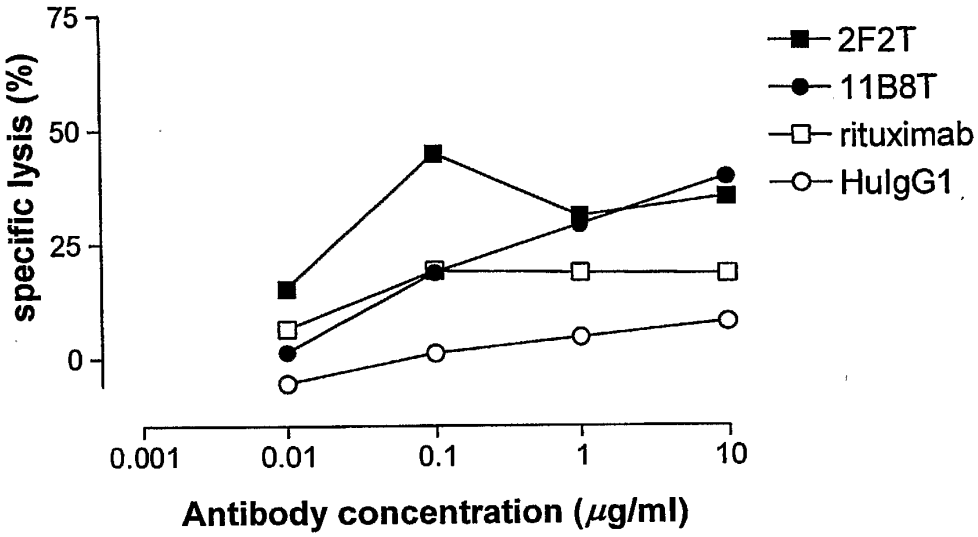


Fig. 28

Samsung Bioepis Exhibit No. 1028
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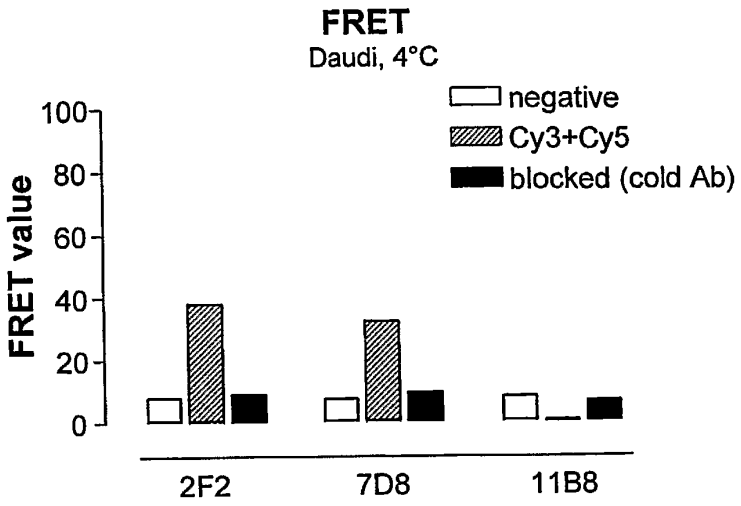


Fig. 29A

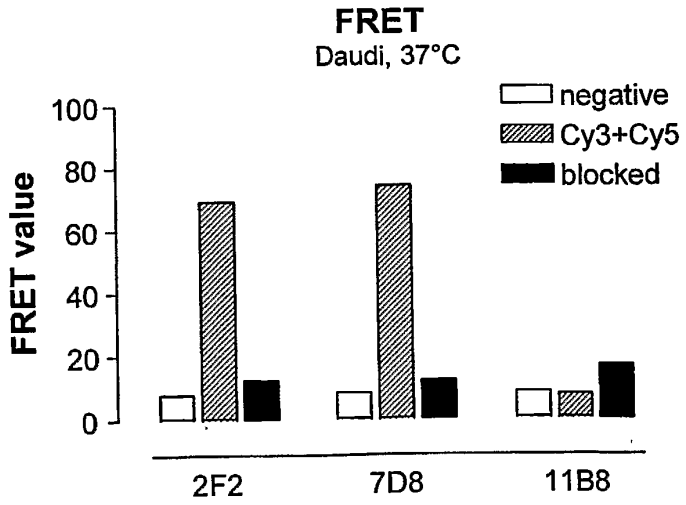


Fig. 29B



Fig. 29C

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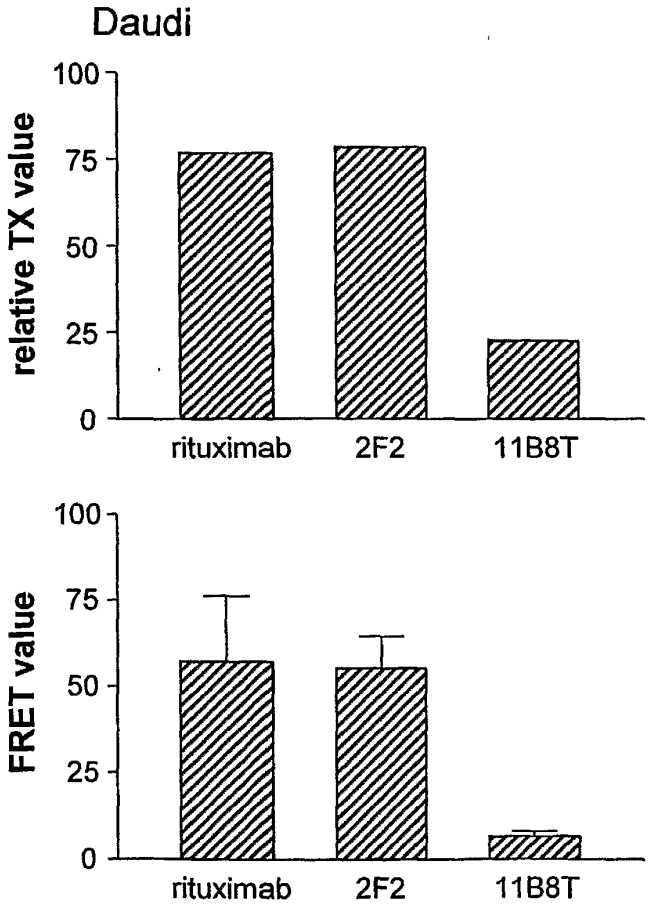

Daudi
relative TX value
100
75
50
25
0
rituximab
2F2
11B8T
FRET value
100
75
50
25
0
rituximab
2F2
11B8T

Fig. 30

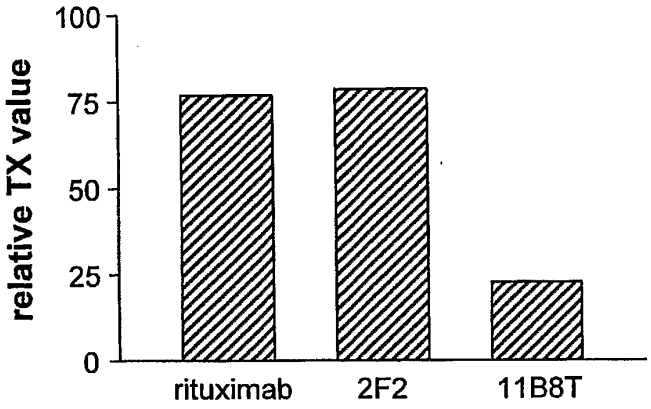


Fig. 31

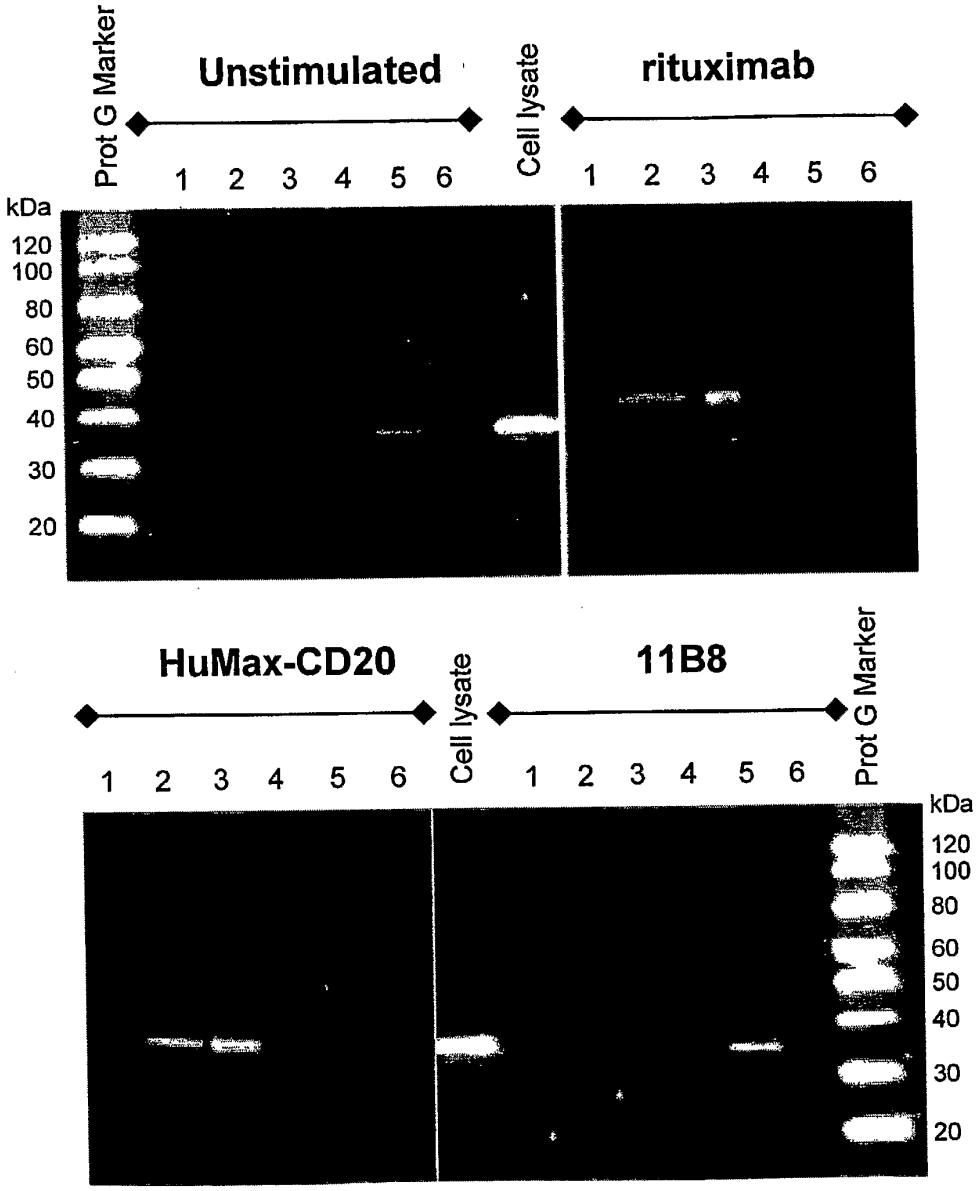

Prot G Marker
Unstimulated
Cell lysate
rituximab
1 2 3 4 5 6
1 2 3 4 5 6
kDa
120
100
80
60
50
40
30
20
HuMax-CD20
Cell lysate
11B8
Prot G Marker
1 2 3 4 5 6
1 2 3 4 5 6
kDa
120
100
80
60
50
40
30
20

Fig. 32

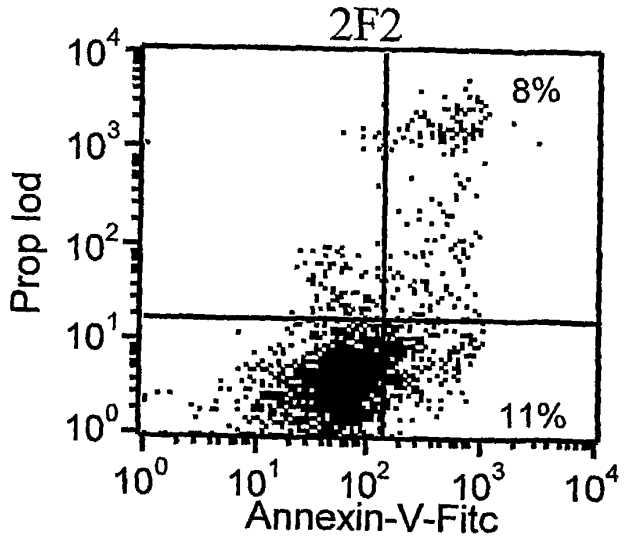


Fig. 33A

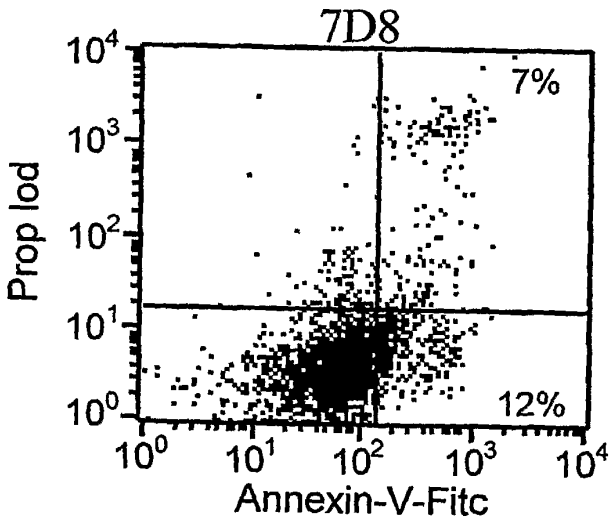


Fig. 33B

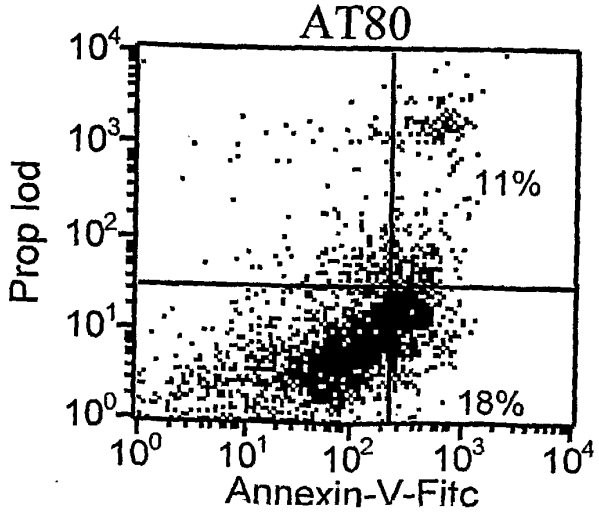


Fig. 33C

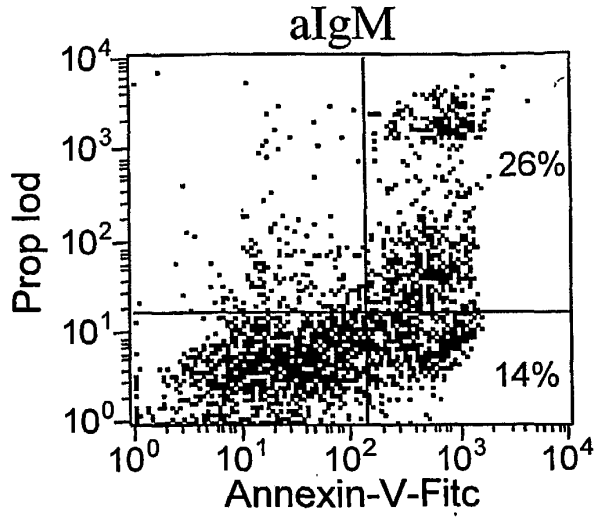


Fig. 33D

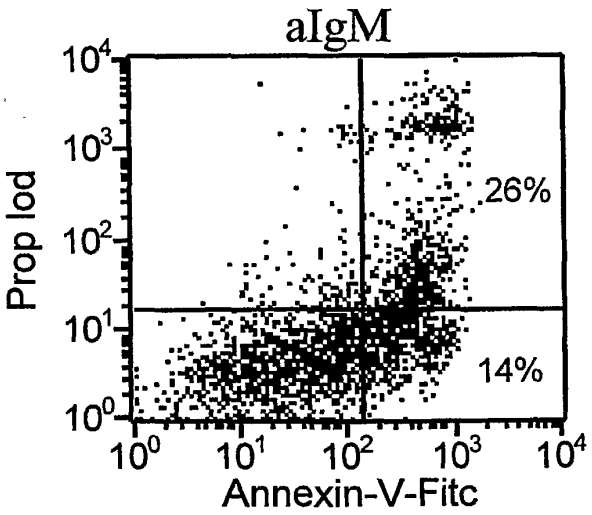


Fig. 33E

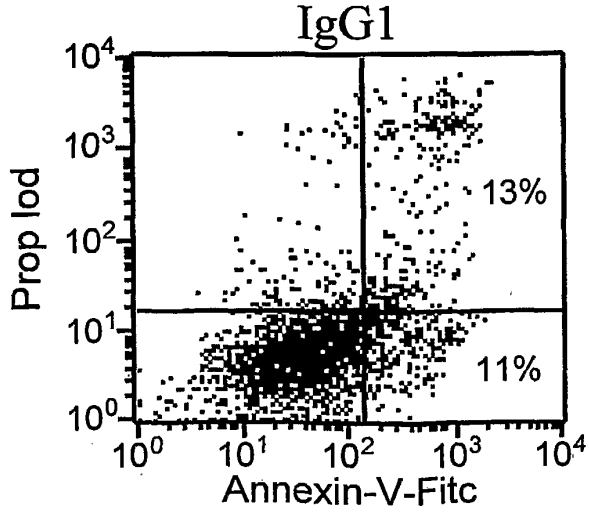


Fig. 33F

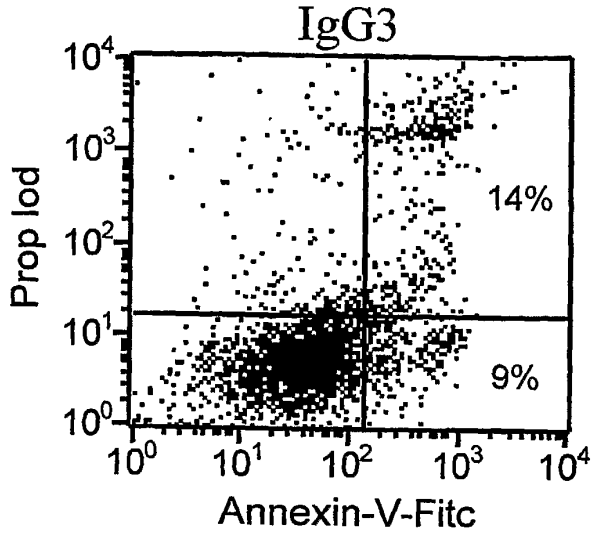


Fig. 33G

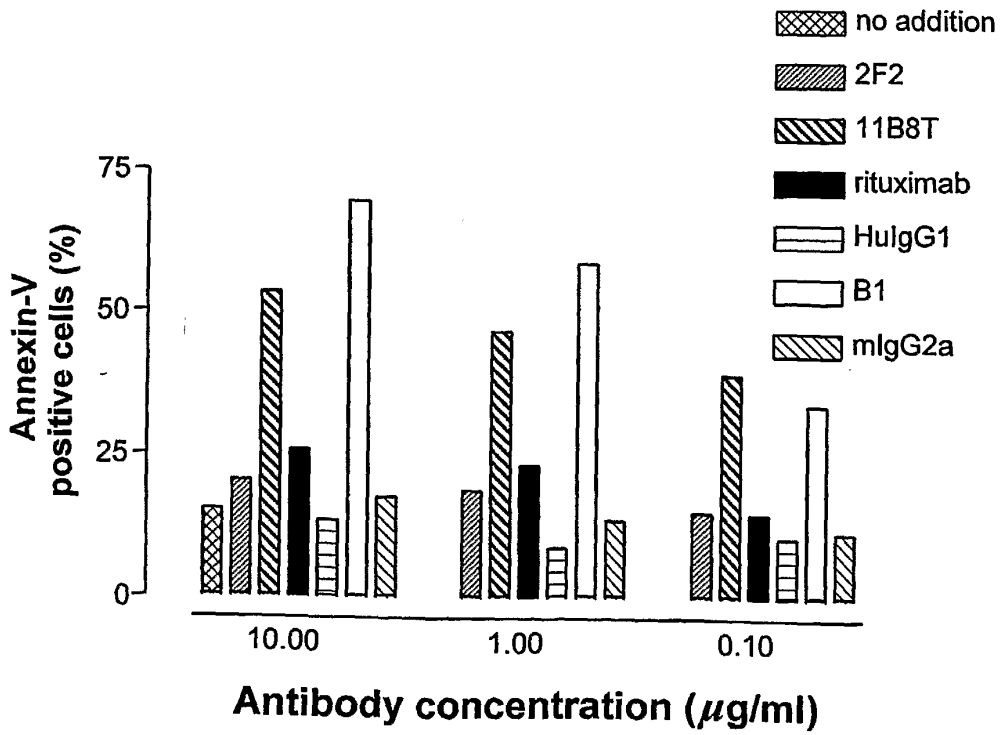


Fig. 34

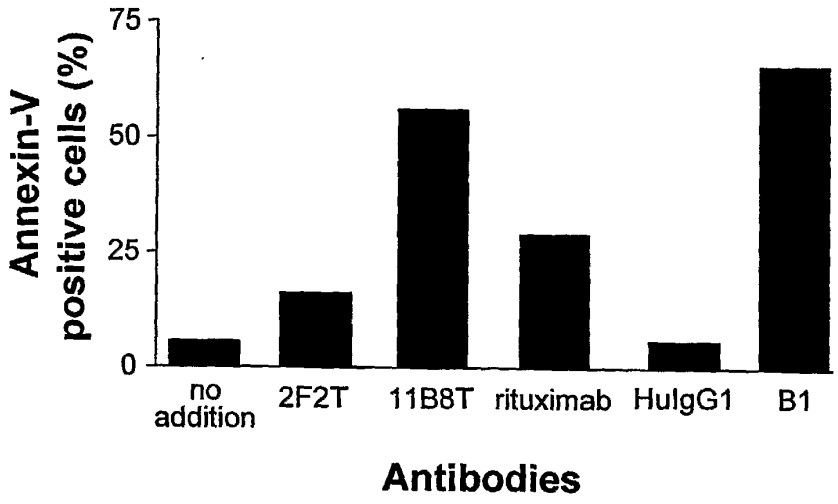

Annexin-V positive cells (%)
75
50
25
0
no addition
2F2T
11B8T
rituximab
HuIgG1
B1
Antibodies

Fig. 35A

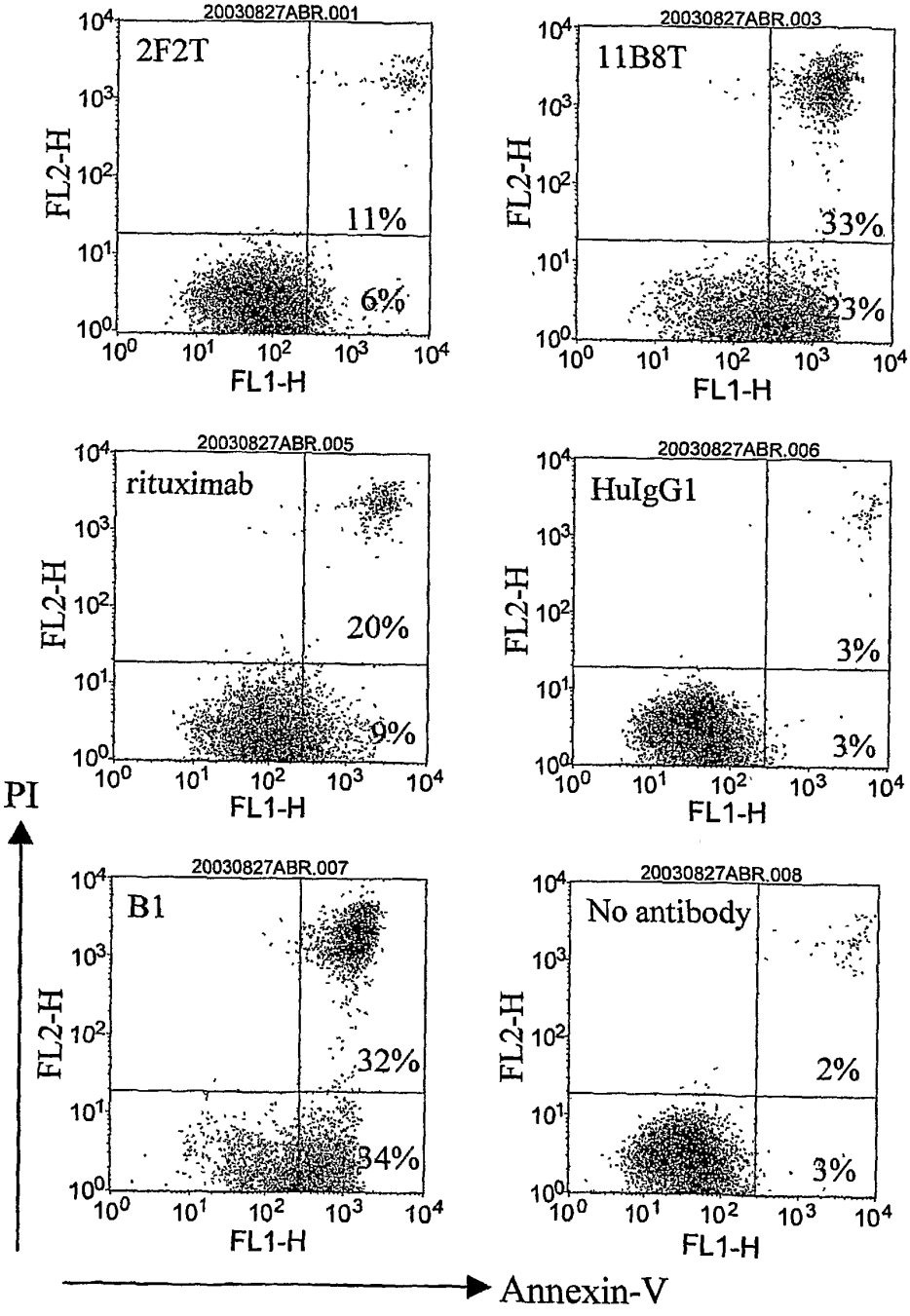

20030827ABR.001
2F2T
11%
6%
20030827ABR.003
11B8T
33%
23%
20030827ABR.005
rituximab
20%
9%
20030827ABR.006
HuIgG1
3%
3%
20030827ABR.007
B1
32%
34%
20030827ABR.008
No antibody
2%
3%
FL2-H
FL1-H
PI
Annexin-V

Fig. 35B

Homotypic adhesion of Ramos cells.

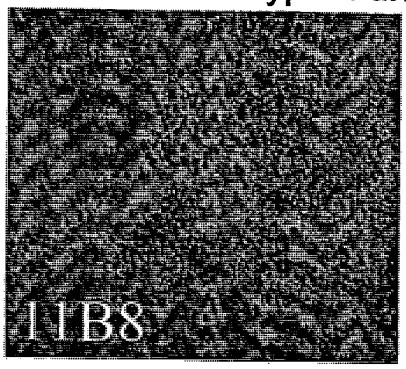


Fig. 36A

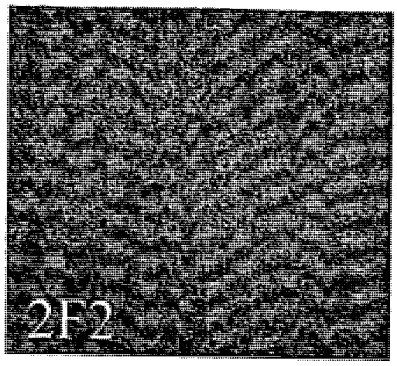


Fig. 36B

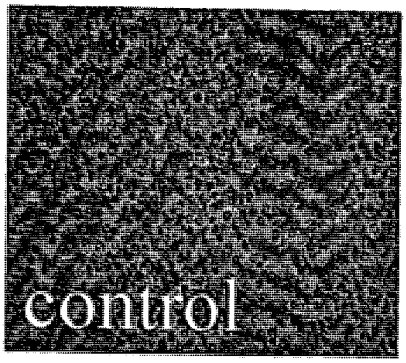


Fig. 36C

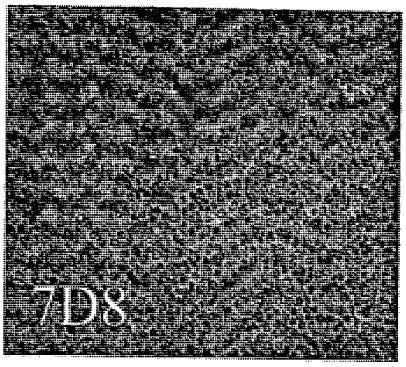


Fig. 36D

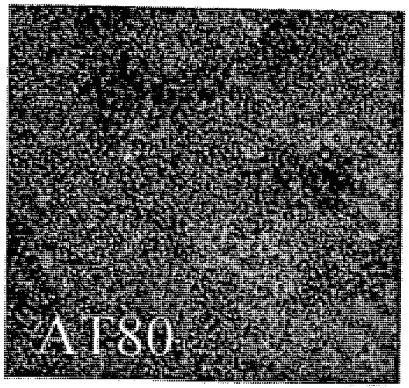


Fig. 36E

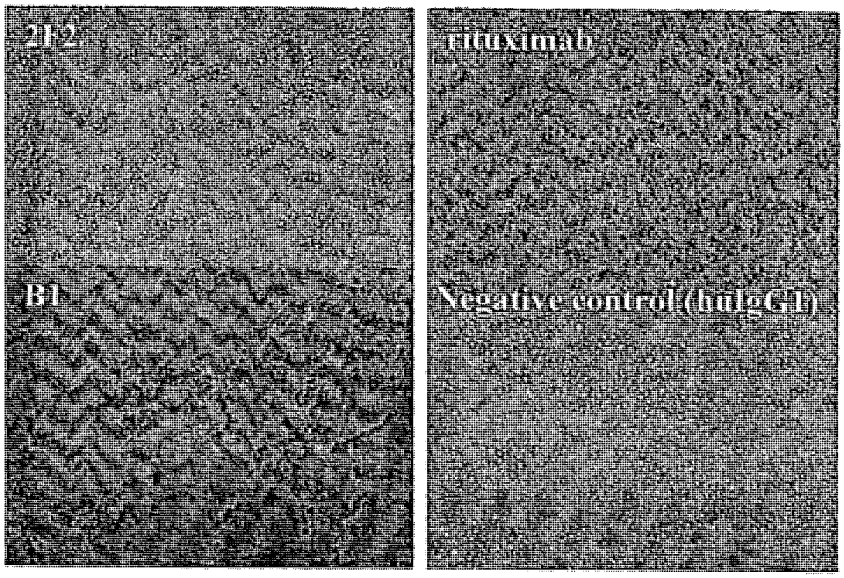


Fig. 37

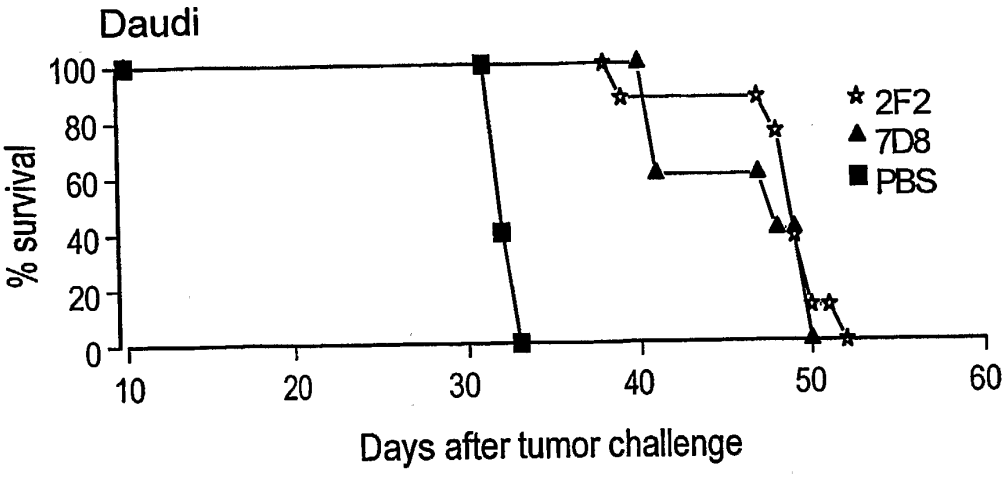


Fig. 38

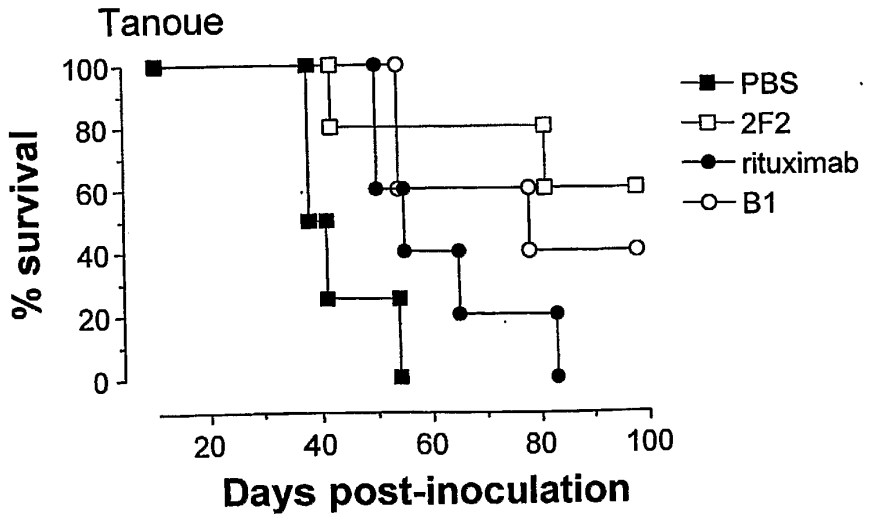


Fig. 39

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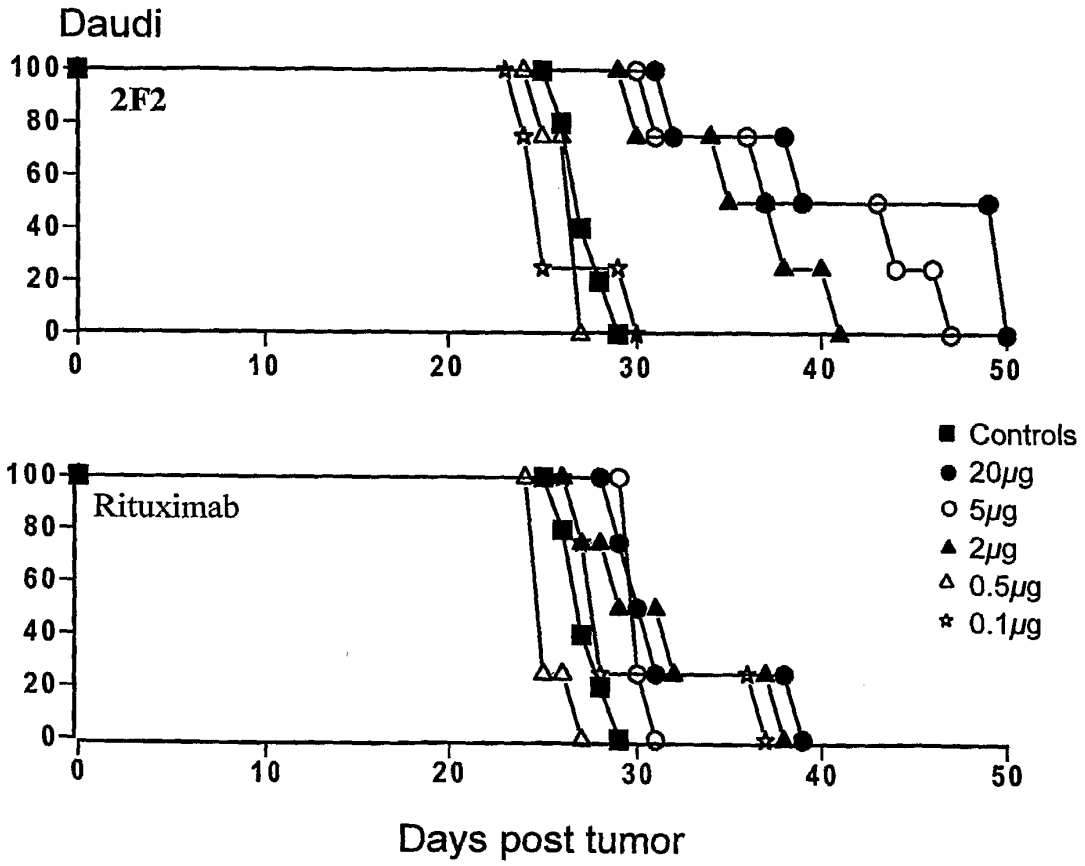

Daudi
2F2
Rituximab
100
80
60
40
20
0
0
10
20
30
40
50
Controls
20μg
5μg
2μg
0.5μg
0.1μg
Days post tumor

Fig. 40

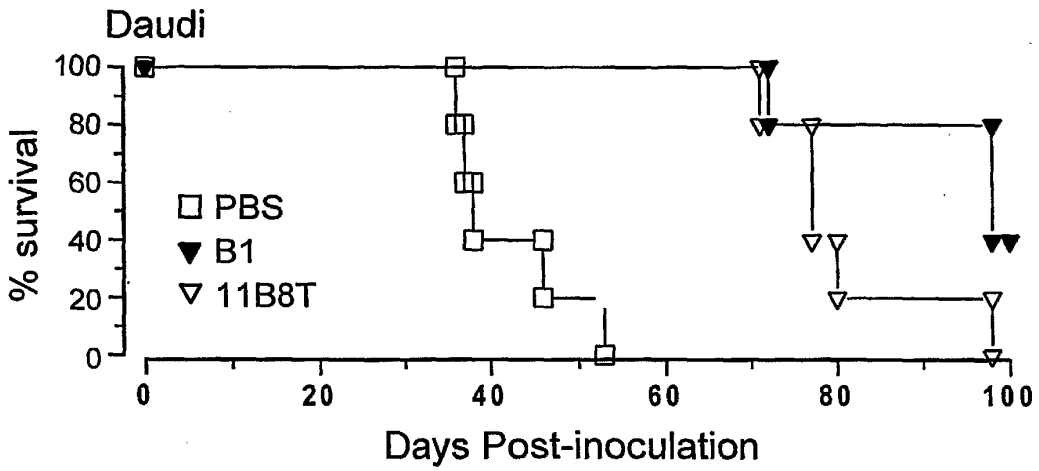

Daudi
100
80
60
40
20
0
% survival
PBS
B1
11B8T
0
20
40
60
80
100
Days Post-inoculation

Fig. 41

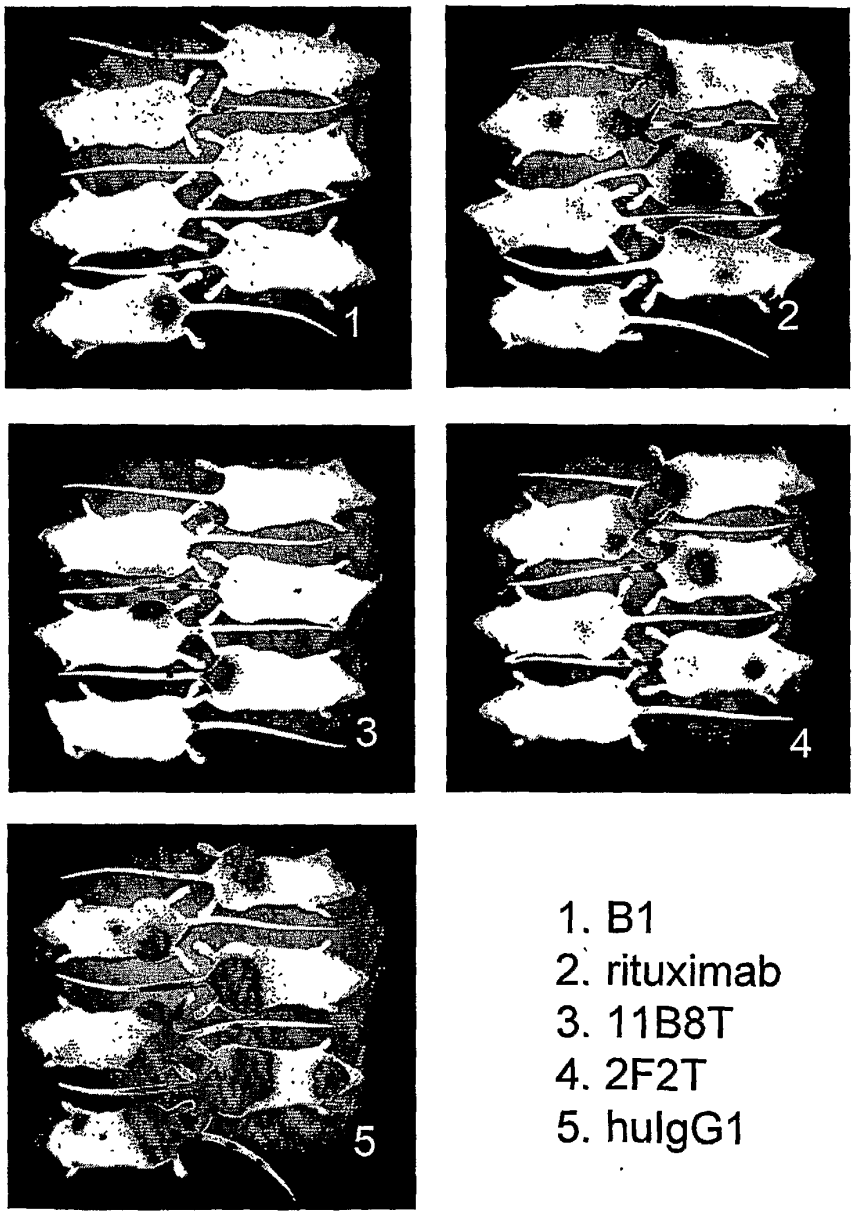


Fig. 42

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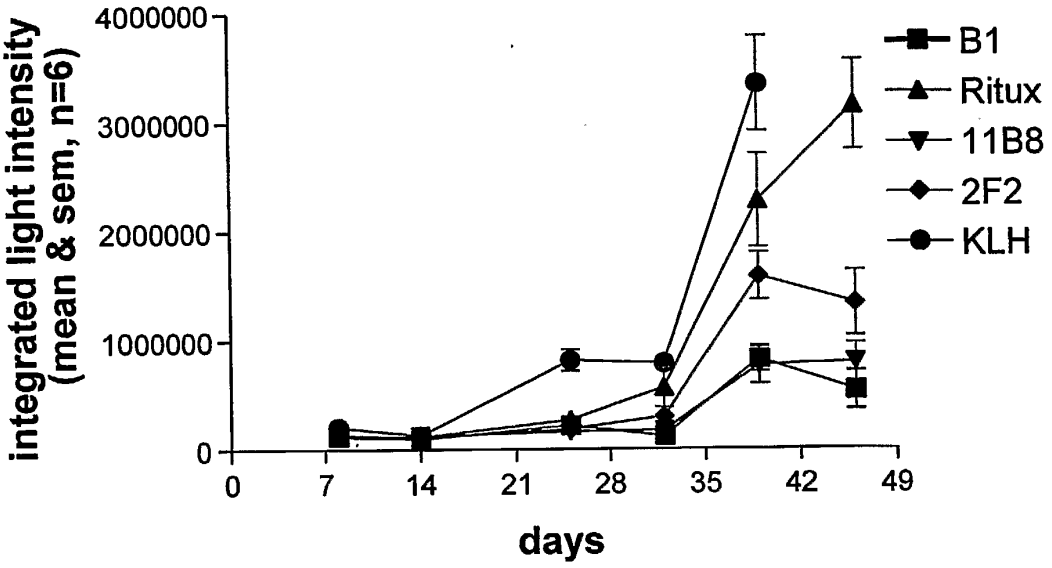


Fig. 43

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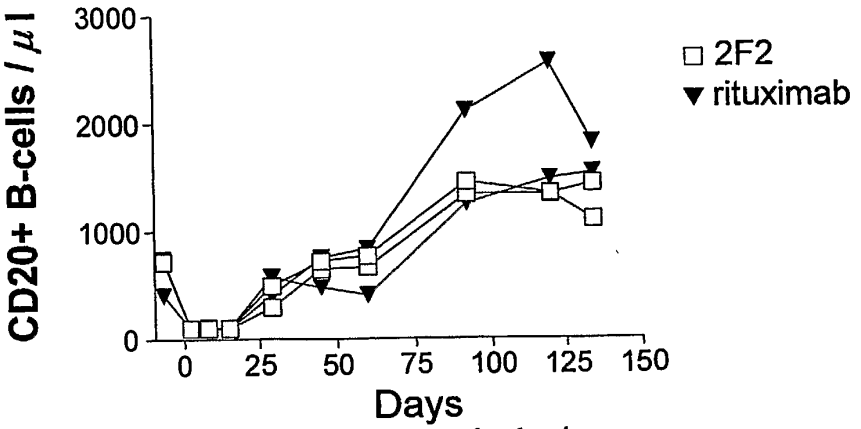


Fig. 44A

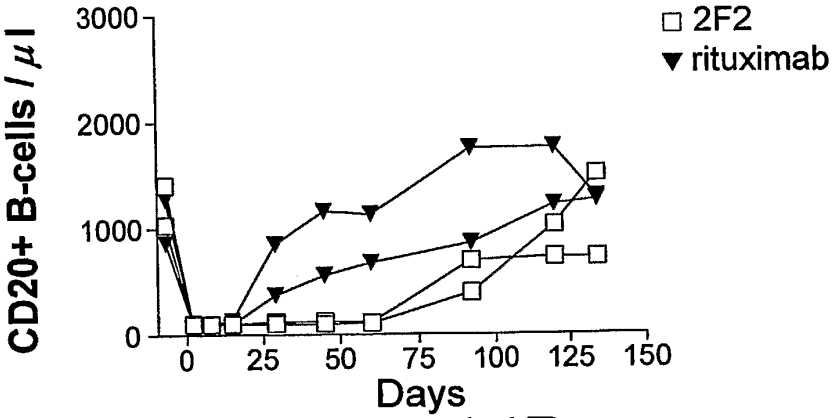


Fig. 44B

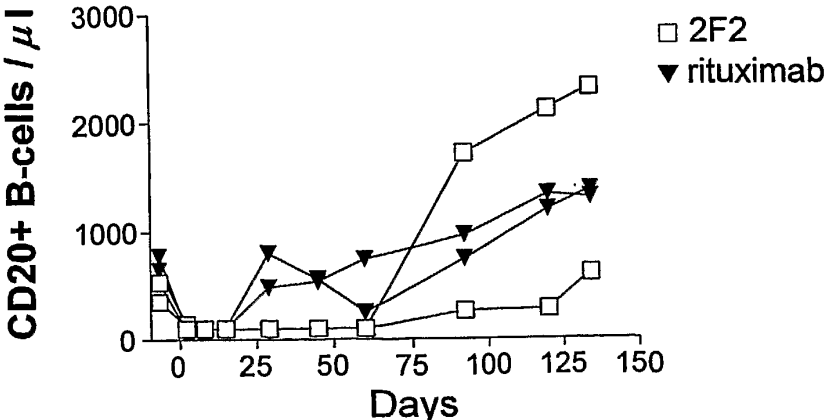


Fig. 44C

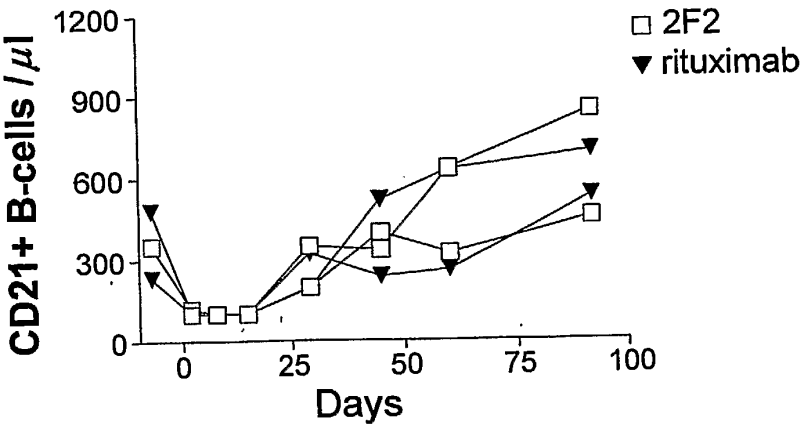


Fig. 45A

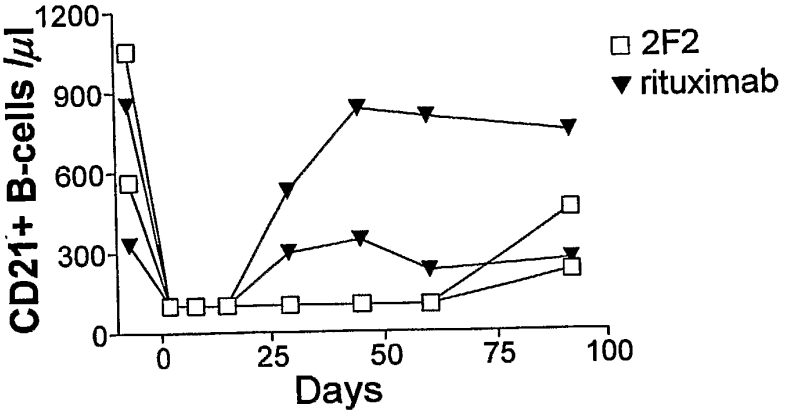


Fig. 45B

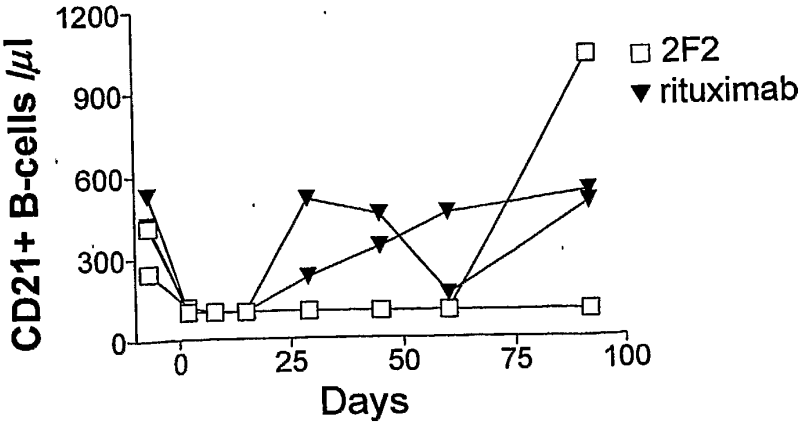


Fig. 45C

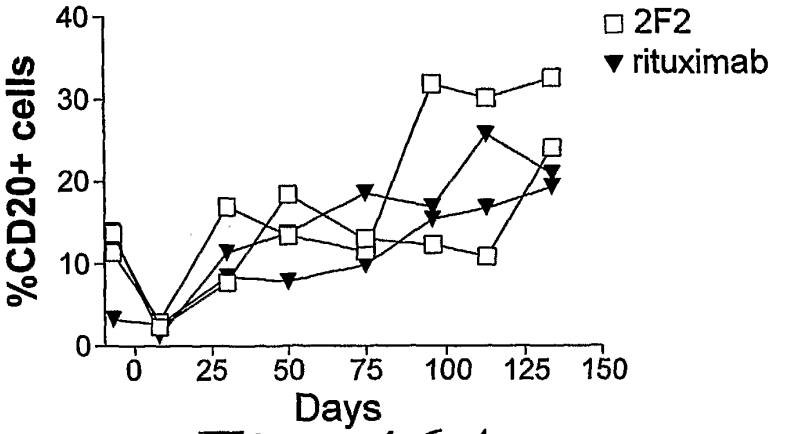


Fig. 46A

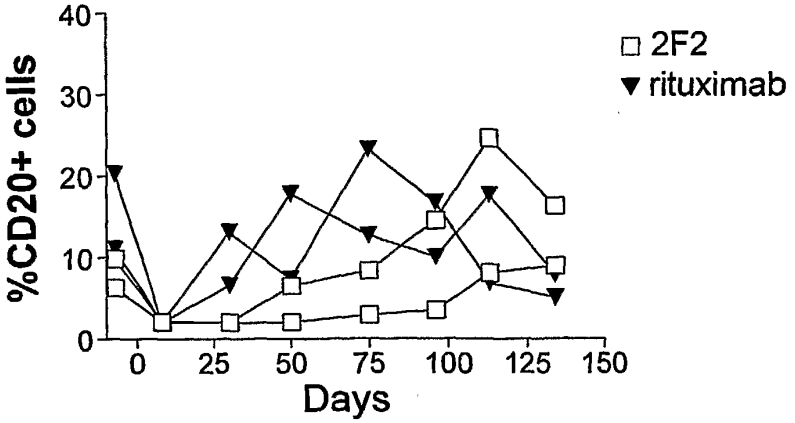


Fig. 46B

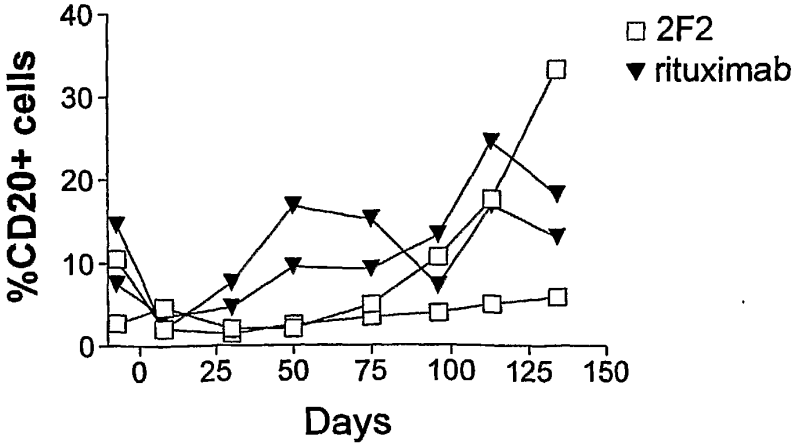


Fig. 46C

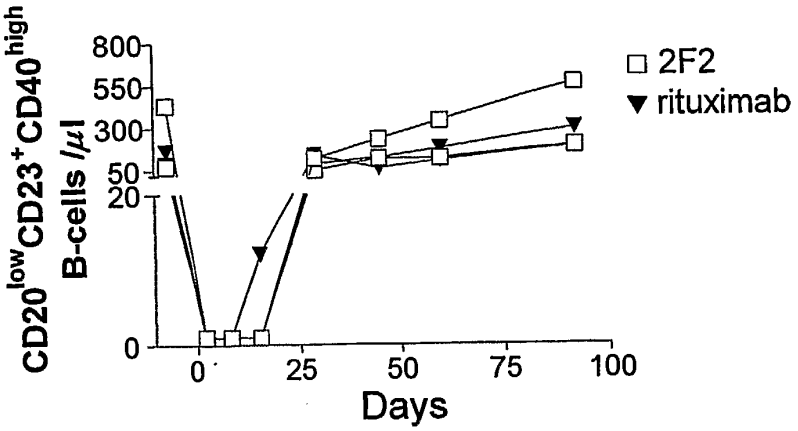


Fig. 47B

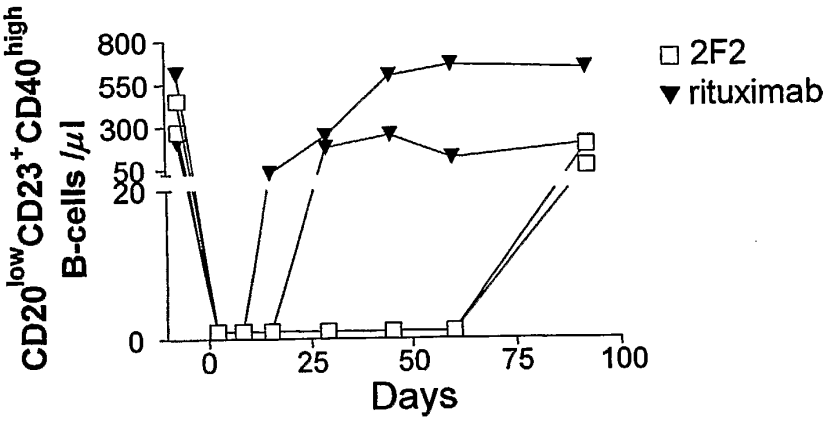


Fig. 47B

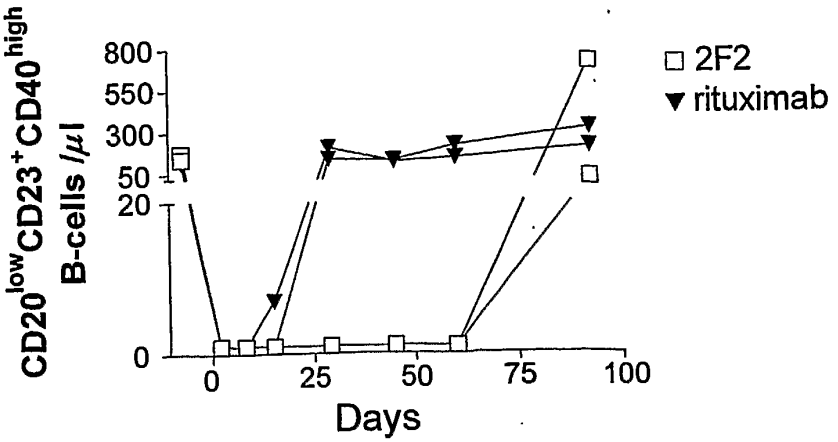


Fig. 47C

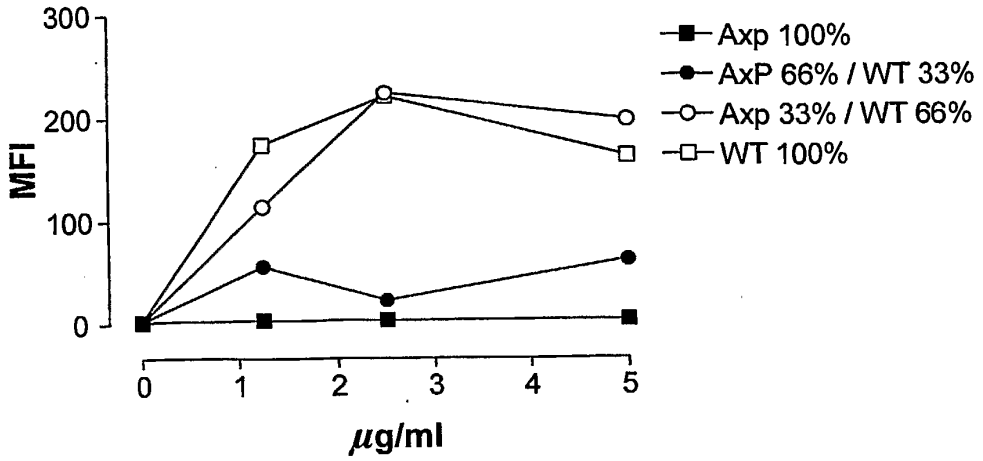


Fig. 48A

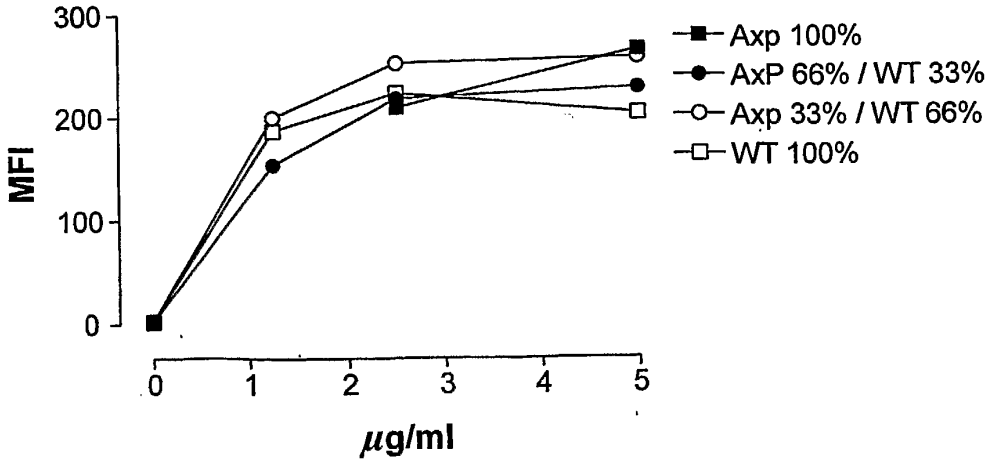


Fig. 48B

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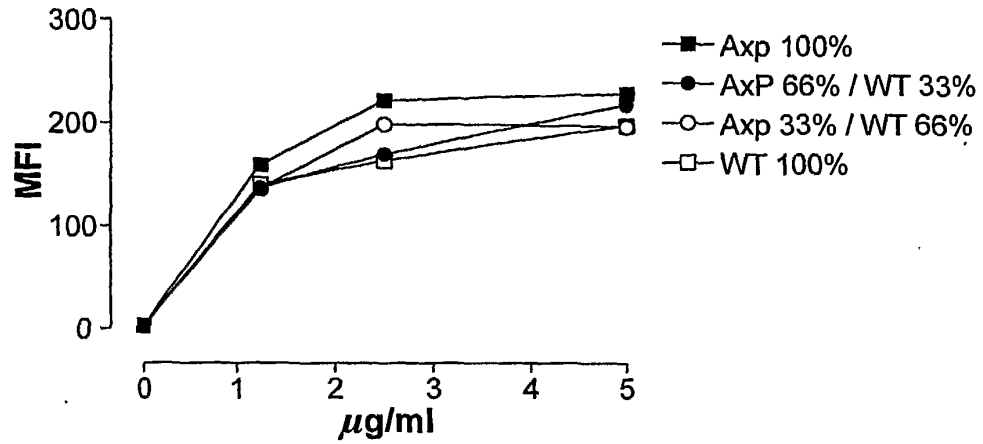


Fig. 48C

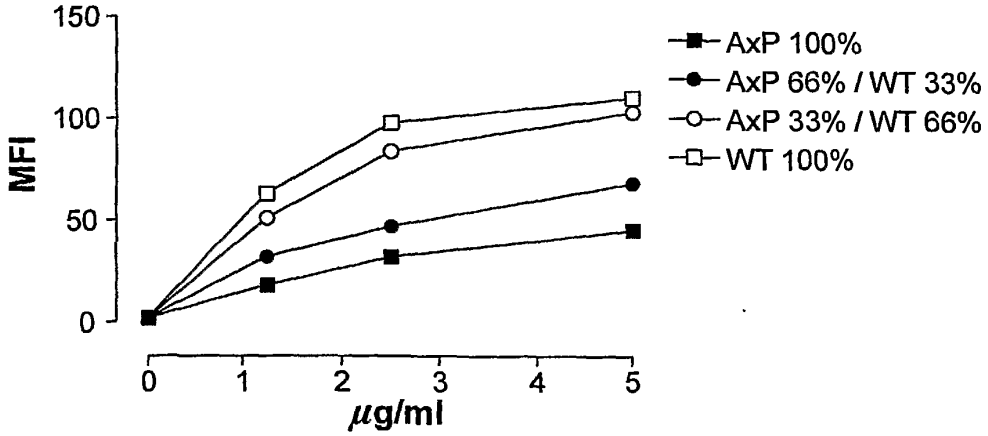


Fig. 48D

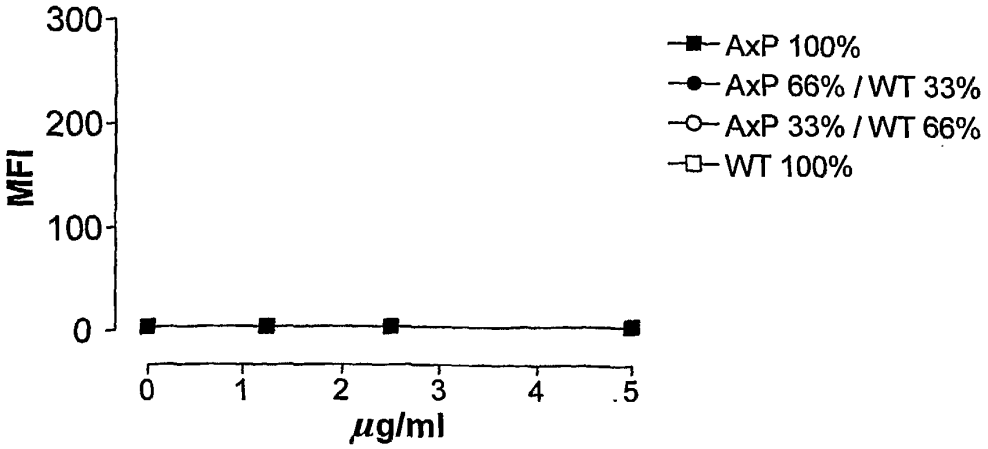


Fig. 48E

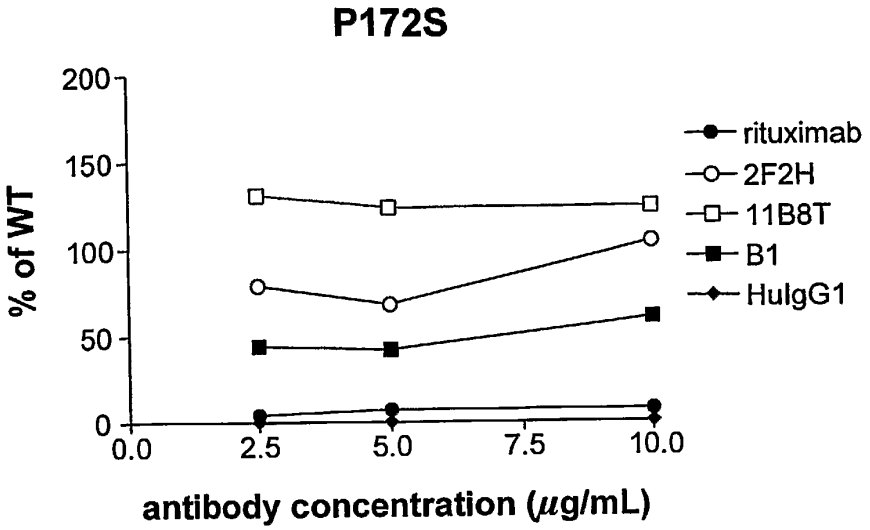


Fig. 49A

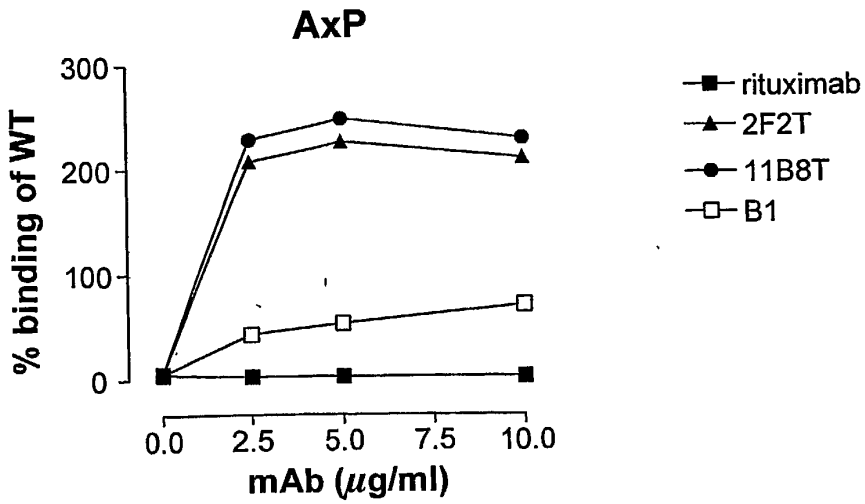


Fig. 49B

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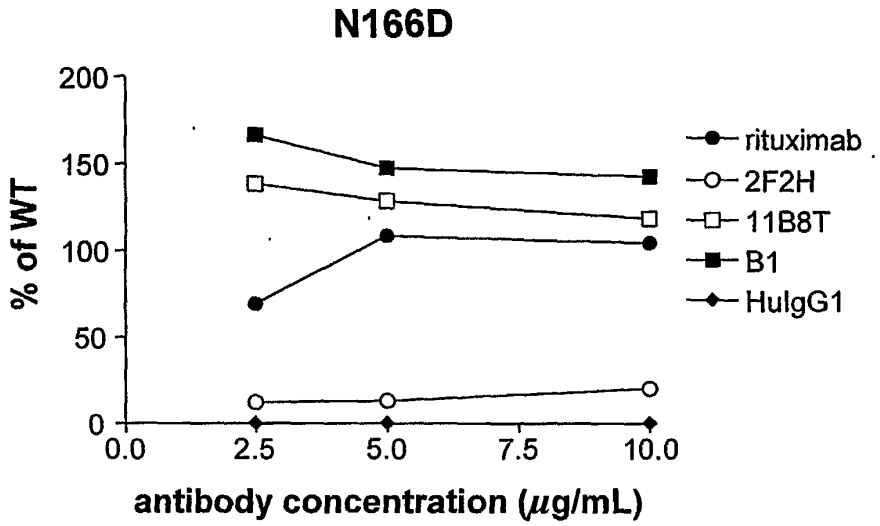


Fig. 49C

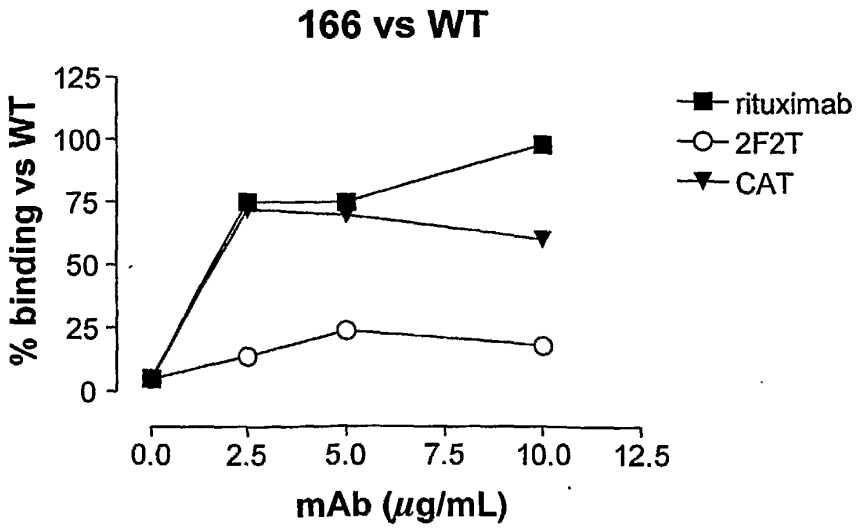


Fig. 49D

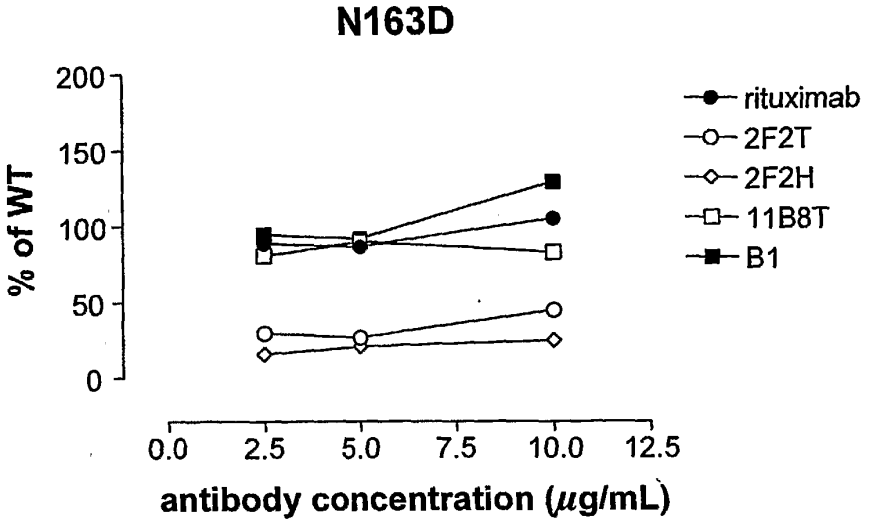


Fig. 49E

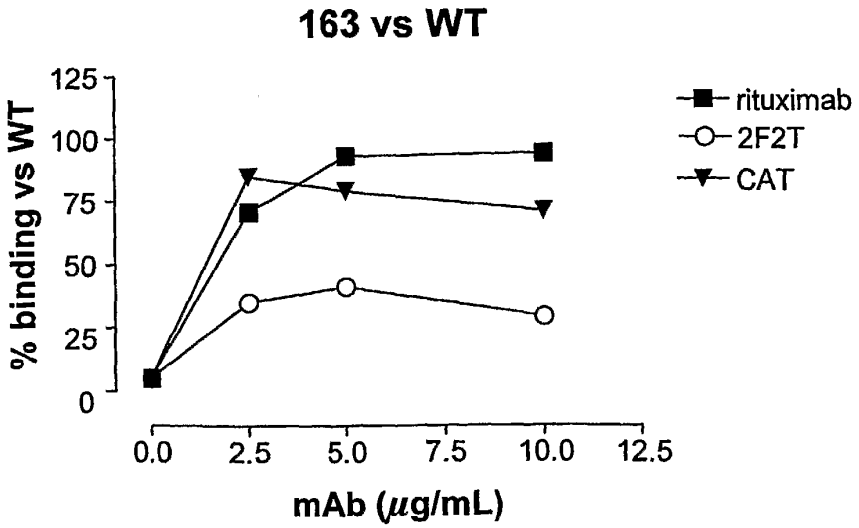


Fig. 49F

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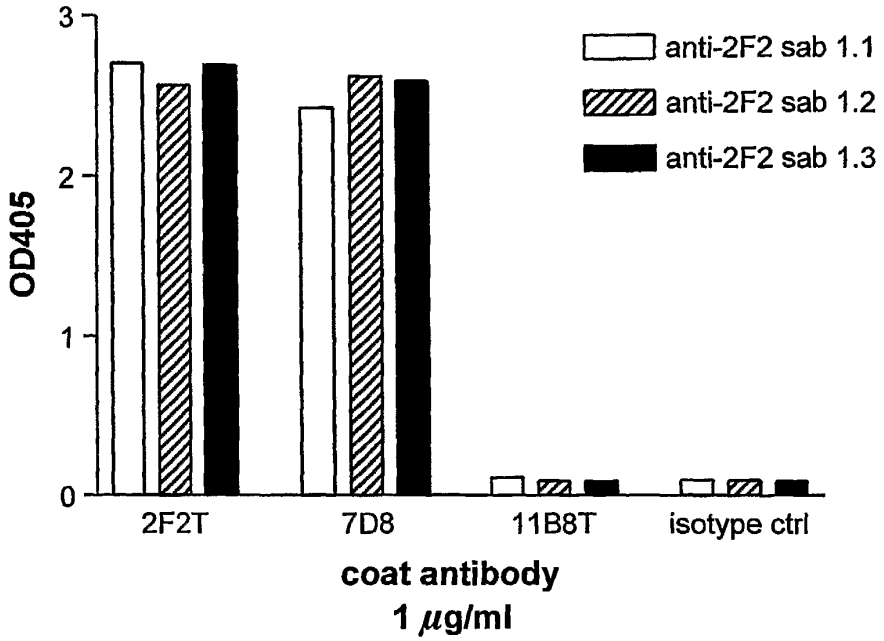


Fig. 50

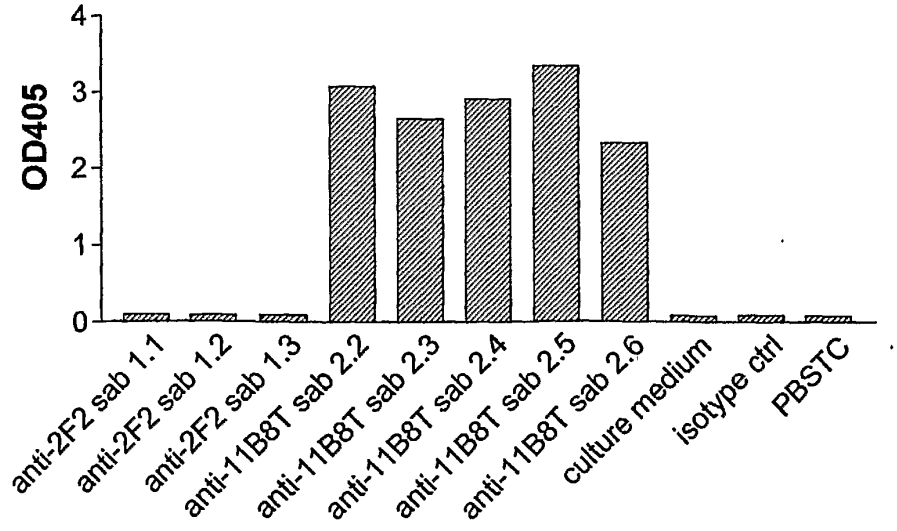


Fig. 51

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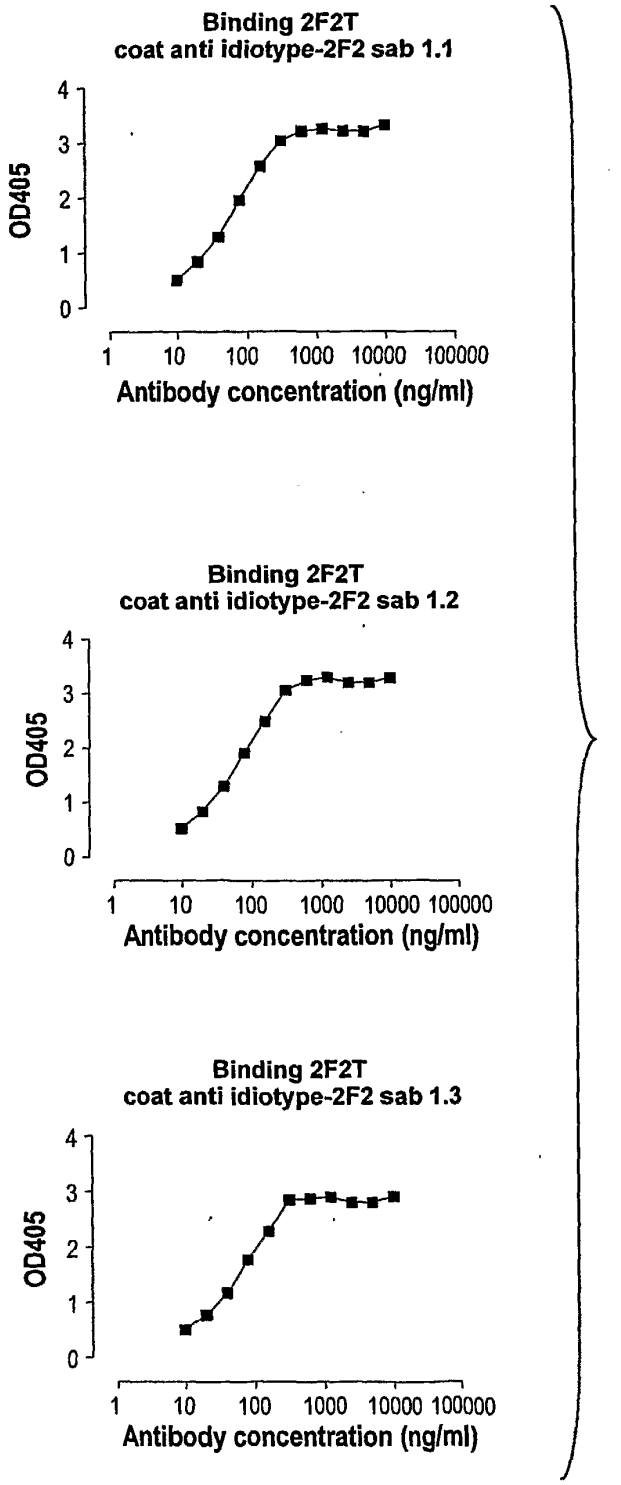


Fig. 52

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Translation of 2F2 VH

```
  1  MFLGLSWIFL LAILKGVQCE VQLVESGGGL VQPGRSLRLS CAASGFTFND
 51  YAMHWVRQAP GKGLEWVSTI SWNSGSIGYA DSVKGRFTIS RDNAKKSLYL
101  QMNSLRAEDT ALYYCAKDIQ YGNYYYGMDV WGQGTTVTVS S
```

Translation of 2F2VL

```
  1  MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51  SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101  EDFAVYYCQQ RSNWPITFGQ GTRLEIK
```

CDR1

CDR2

CDR3

Fig. 53

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2F2 VH

```
1   ATGGAG TTGGGA CTGAGC TGGATT TTCCTT TTGGCT ATTTTA AAAGGT GTCCAG
55  TGTGAA GTGCAG CTGGTG GAGTCT GGGGGA GGCTTG GTACAG CCTGGC AGGTCC
109 CTGAGA CTCTCC TGTGCA GCCTCT GGATTC ACCTTT AATGAT TATGCC ATGCAC
163 TGGGTC CGGCAA GCTCCA GGGAAG GGCCTG GAGTGG GTCTCA ACTATT AGTTGG
217 AATAGT GGTTCC ATAGGC TATGCG GACTCT GTGAAG GGCCGA TTCACC ATCTCC
271 AGAGAC AACGCC AAGAAG TCCCTG TATCTG CAAATG AACAGT CTGAGA GCTGAG
325 GACACG GCCTTG TATTAC TGTGCA AAAGAT ATACAG TACGGC AACTAC TACTAC
379 GGTATG GACGTC TGGGGC CAAGGG ACCACG GTCACC GTCTCC TCAG
```

2F2VL

```
1   ATGGAA GCCCCA GCTCAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
109 GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
163 TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
217 AACAGG GCCACT GGCATC CCAGCC AGGTTC CGTGGC AGTGGG TCTGGG ACAGAC
271 TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
325 CAGCAG CGTAGC AACTGG CCGATC ACCTTC GGCCAA GGGACA CGACTG GAGATT
379 AAAC
```

Fig. 54

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Translation of 7D8VH

```
  1   MELGLSWIFL LAILKGVQCE VQLVESGGGL VQPDRSLRLS CAASGFTFHD
 51   YAMHWVRQAP GKGLEWVSTI SWNSGTIGYA DSVKGRFTIS RDNAKNSLYL
101   QMNSLRAEDT ALYYCAKDIQ YGNYYYGMDV WGQGTTVTVS S
```

Translation of 7D8VL

```
  1   MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51   SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101   EDFAVYYCQQ RSNWPTTFGQ GTRLEIK
```

CDR1

CDR2

CDR3

Fig. 55

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7D8VH

```
   1  ATGGAG TTGGGA CTGAGC TGGATT TTCCTT TTGGCT ATTTTA AAAGGT GTCCAG
  55  TGTGAA GTGCAG CTGGTG GAGTCT GGGGGA GGCTTG GTACAG CCTGAC AGGTCC
 109  CTGAGA CTCTCC TGTGCA GCCTCT GGATTC ACCTTT CATGAT TATGCC ATGCAC
 163  TGGGTC CGGCAA GCTCCA GGGAAG GGCCTG GAGTGG GTCTCA ACTATT AGTTGG
 217  AATAGT GGTACC ATAGGC TATGCG GACTCT GTGAAG GGCCGA TTCACC ATCTCC
 271  AGAGAC AACGCC AAGAAC TCCCTG TATCTG CAAATG AACAGT CTGAGA GCTGAG
 325  GACACG GCCTTG TATTAC TGTGCA AAAGAT ATACAG TACGGC AACTAC TACTAC
 379  GGTATG GACGTC TGGGGC CAAGGG ACCACG GTCACC GTCTCC TCAG
```

7D8VH

```
   1  ATGGAA GCCCCA GCTCAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
  55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
 109  GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
 163  TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
 217  AACAGG GCCACT GGCATC CCAGCC AGGTTC AGTGGC AGTGGG TCTGGG ACAGAC
 271  TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
 325  CAGCAG CGTAGC AACTGG CCGATC ACCTTC GGCCAA GGGACA CGACTG GAGATT
 379  AAAC
```

Fig. 56

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Translation of VHCD2011B8

```
  1   MELGLSWVFL VAILKGVQCE VQLVQSGGGL VHPGGSLRLS CTGSGFTFSY
 51   HAMHWVRQAP GKGLEWVSII GTGGVTYYAD SVKGRFTISR DNVKNSLYLQ
101   MNSLRAEDMA VYYCARDYYG AGSFYDGIYG MDYWGQGTTV TVSS
```

Translation of VLCD2011B8

```
  1   MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS
 51   SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP
101   EDFAVYYCQQ RSDWPLTFGG GTKVEIK
```

CDR1

CDR2

CDR3

Fig. 57

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VHCD2011B8

```
   1  ATGGAG TTGGGG CTGAGC TGGGTT TTCCTT GTTGCT ATATTA AAAGGT GTCCAG
  55  TGTGAG GTTCAG CTGGTG CAGTCT GGGGGA GGCTTG GTACAT CCTGGG GGGTCC
 109  CTGAGA CTCTCC TGTACA GGCTCT GGATTC ACCTTC AGTTAC CATGCT ATGCAT
 163  TGGGTT CGCCAG GCTCCA GGAAAA GGTCTG GAATGG GTATCA ATTATT GGGACT
 217  GGTGGT GTCACA TACTAT GCAGAC TCCGTG AAGGGC CGATTC ACCATC TCCAGA
 271  GACAAT GTCAAG AACTCC TTGTAT CTTCAA ATGAAC AGCCTG AGAGCC GAGGAC
 325  ATGGCT GTGTAT TACTGT GCAAGA GATTAC TATGGT GCGGGG AGTTTT TATGAC
 379  GGCCTC TACGGT ATGGAC GTCTGG GGCCAA GGGACC ACGGTC ACCGTC TCCTCA
 433  G
```

VLCD2011B8

```
   1  ATGGAA GCCCCA GCACAG CTTCTC TTCCTC CTGCTA CTCTGG CTCCCA GATACC
  55  ACCGGA GAAATT GTGTTG ACACAG TCTCCA GCCACC CTGTCT TTGTCT CCAGGG
 109  GAAAGA GCCACC CTCTCC TGCAGG GCCAGT CAGAGT GTTAGC AGCTAC TTAGCC
 163  TGGTAC CAACAG AAACCT GGCCAG GCTCCC AGGCTC CTCATC TATGAT GCATCC
 217  AACAGG GCCACT GGCATC CCAGCC AGGTTC AGTGGC AGTGGG TCTGGG ACAGAC
 271  TTCACT CTCACC ATCAGC AGCCTA GAGCCT GAAGAT TTTGCA GTTTAT TACTGT
 325  CAGCAG CGTAGC GACTGG CCGCTC ACTTTC GGCGGA GGGACC AAGGTG GAGATC
 379  AAAC
```

Fig. 58

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GMI-055PC

SEQUENCE LISTING

<110> Genmab, Inc. *et al.*

<120> HUMAN MONOCLONAL ANTIBODIES AGAINST CD20

<130> GMI-055PC

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<151> 2002-10-17

<150> US 60/460,028
<151> 2002-04-02

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tgtgcagcct ctggattcac ctttaatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggttccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaagtc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
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tcag 424

<210> 2
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<212> PRT
<213> Homo sapiens

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 20 25 30
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 35 40 45
Asn Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 50 55 60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Ser Ile Gly Tyr Ala
65 70 75 80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys
 85 90 95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
 100 105 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
 115 120 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
 130 135 140

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Page 1210

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<212> DNA
<213> Homo sapiens

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ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
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aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
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gggacacgac tggagattaa ac 382

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 35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
 50 55 60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65 70 75 80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
 85 90 95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
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 115 120 125

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<211> 424
<212> DNA
<213> Homo sapiens

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Pro Asp Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50 55 60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65 70 75 80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
85 90 95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
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115 120 125
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130 135 140

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aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
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gggacacgac tggagattaa ac 382

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35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
50 55 60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65 70 75 80

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```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
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            20                  25                  30
Pro Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly Ser Gly Phe Thr Phe
        35                  40                  45
Ser Tyr His Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp
65                  70                  75                  80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Val Lys Asn Ser
                85                  90                  95
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr
            100                 105                 110
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        115                 120                 125
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ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
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<212> PRT
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20 25 30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
35 40 45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
50 55 60
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Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
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        35                  40                  45
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    50                  55                  60
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GMI-055PC

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            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
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20 July 2011

Dear Sirs

European Patent Application No. 03809145.0-2402
GENMAB A/S
Our Ref: N.94565 GCW/no

Thank you for the Communication under Rule 71(3) EPC dated 31 March 2011. As a preliminary response, I kindly request amendment of the application as follows:

- please replace pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44 at present on file by correspondingly-numbered new pages provided;
- please replace pages 1 to 7 of the sequence listing by the correspondingly-numbered new sequence listing pages provided; and
- please replace pages 110 and 111 at present on file by the correspondingly-numbered new pages provided.

New pages 2, 3 and 31 have been retyped. I therefore also attach a hand-amended version of original pages 2, 3 and 31, i.e of pages 2, 3 and 31 of the description of the original International Application Publication No. WO 2004/035607, showing how the new pages have been derived. Similarly, I attach a hand-amended version of original pages 1 to 7 of the sequence listing showing how the new sequence listing pages have been derived.

New Pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44

I am filing replacement pages 2 to 8, 13, 14, 21, 31, 32, 35 and 39 to 44 in order to conform the description more precisely to the invention now claimed. I wish to avoid any possible difficulties

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□ *Solicitor*

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and ambiguities in interpretation of the granted claims.

The description which was amended by the Examining Division to derive the description of the Druckexemplar was in fact the description on file on the basis of the response filed 23 February 2007 to the first Examination Report dated 28 April 2006. In that response, replacement pages 2, 3, 3a to 3c, 5, 6, 6a, 8, 32 and 35 were submitted in order to bring the description into conformity with the replacement claims that were being filed at that time. Exact counterparts to some of those claims were therefore included in the description.

However, the claims which have now been allowed and which are incorporated in the Druckexemplar are rather different from the claims submitted in response to the first Examination Report. It is therefore inappropriate to retain in the description the passages which were added on 23 February 2007 in the response to the first Examination Report to provide counterparts to the claims being filed at that time. Indeed, the presence of these passages risks causing confusion about the interpretation of the claims.

In order to derive the new pages of the description which are being filed now, I have returned to the original pages of the description, i.e. to the pages of the description of WO 2004/035607. I have thus retained the original disclosure wherever possible. I have provided an exact counterpart to allowed claim 1 on new page 2 at the beginning of the *"Summary of the Invention"* section. Subsequent pages have been revised to delete references to antibody 11B8 as an antibody of the invention.

Lines 23 to 34 on page 3 have been deleted because this passage is broader in scope than allowed claim 1. I have retained mention of *"the invention"* and not referred instead to *"the disclosure"*. The relevant passages do still relate to the claimed invention. Further, I wish to avoid problems which might arise post-grant concerning the scope of the invention if the term *"the disclosure"* were to be retained. Thus, I wish to avoid inconsistencies between the claims and the description which could render the scope of the claims unclear.

Correction of Sequence Listing

I request correction under Rule 139 EPC of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 13, 19 and 25 of the sequence listing. These corrections fall into two categories:

- First, I request correction SEQ ID NOs: 2, 4, 6, 8, 10 and 12 to remove the leader sequences that were erroneously included.

- Second, I request correction SEQ ID NOs: 13, 19 and 25 to remove the extra N-terminal amino acid which was erroneously included in each case.

For all of the requested corrections, it is immediately evident that an error has occurred and what the

correction should be (Examination Guidelines C-VI-5.4). In that regard, I attach a copy of a Declaration from Dr Tom Vink, Associate Director, Cell & Molecular Sciences, Genmab BV, and a copy of the attachments to which the Declaration refers. Please see Dr Vink's explanations.

Claims 38 and 40

I wish to clarify claims 38 and 40. These two claims as amended in the Druckexemplar refer to a human antibody, composition, expression vector, immunoconjugate or bispecific molecule "*for the use according to claim 37*" or "*for the use according to claim 39*" respectively. However, claims 37 and 39 are not "*use*" claims.

On a literal interpretation, claims 38 and 40 could now be considered as being directed to any human antibody, composition, expression vector, immunoconjugate or bispecific molecule for use in treating or preventing follicular lymphoma, B cell chronic lymphocytic leukaemia or small lymphocytic lymphoma (claim 38) or rheumatoid arthritis (claim 40). I therefore wish to amend the two claims to avoid any such possible implication whilst retaining the correct claim format for second medical use claims.

Our further response will follow in due course enclosing the corresponding translations of the claims into French and German and a debit order authorising deduction of the necessary fees from our deposit account 2805.0038.

Yours faithfully



G C WOODS

DECLARATION

I, Tom Vink, Associate Director, Cell & Molecular Sciences at Genmab B.V. do hereby declare and state:

1. Since 1 July 2002, I am an employee of Genmab B.V., which is an affiliate of Genmab A/S.

2. I have thorough experience within the fields of molecular biology, protein biochemistry and antibody engineering

3. PCT application No. WO 2004/035607 has been disclosed to me.

4. The application relates to human monoclonal antibodies against CD20, and it exemplifies three antibodies, 2F2, 7D8 and 11B8. The application contains claims relating to human monoclonal anti-CD20 antibodies, wherein the antibodies are characterized by the variable heavy chain (V_H) and variable light chain (V_L) amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12), cf. claims 15, 17 and 19 of the application:

	2F2	**7D8**	**11B8**
V_H nucleotide	SEQ ID NO:1	SEQ ID NO:5	SEQ ID NO:9
V_L nucleotide	SEQ ID NO:3	SEQ ID NO:7	SEQ ID NO:11
V_H amino acid	SEQ ID NO:2	SEQ ID NO:6	SEQ ID NO:10
V_L amino acid	SEQ ID NO:4 (identical to SEQ ID NO:8)	SEQ ID NO:8 (identical to SEQ ID NO:4)	SEQ ID NO:12

5. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the leader sequences erroneously are included in the variable heavy chain and light chain amino acid sequences (SEQ ID Nos: 2, 6, 10, 4, 8 and 12) which define the mature antibodies 2F2, 7D8 and 11B8, respectively, and (ii) in the affirmative, how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

6. For the reasons explained in the following I believe it would have been obvious for a person skilled in the art at the date of the earliest priority date on 17 October 2002 (i) that the leader sequences (marked in red in Appendix A attached hereto) are

included in the variable heavy chain and light chain amino acid sequences, and (ii) how the variable heavy chain and light chain amino acid sequences without leader sequences should read.

7. It is well known that antibodies are secretory proteins, i.e. proteins that are transported to the extracellular medium by passing through the intracellular secretory pathway of the cell, the first compartment of which is the endoplasmic reticulum. Like other secretory proteins, an antibody heavy or light chain protein is initially produced as a precursor polypeptide containing a so called signal or leader peptide (also denoted leader sequence), which is necessary to direct the polypeptide into the endoplasmic reticulum for further transport and secretion. As for other soluble secretory proteins, during the transport into the endoplasmic reticulum, the signal peptides of the antibody heavy and light chains are cleaved off, resulting in a final mature protein (see also page 29, lines 35-36 of the application). In conclusion, any heavy or light chain from an antibody produced by e.g. a hybridoma will be derived from DNA and RNA constructs in which the heavy and light chain encoding sequences are immediately preceded by the leader sequences.

8. To determine the cleavage site between the leader sequence and the antibody sequence so as to determine where the leader sequence stops and where the antibody sequence starts, a comparison can be made with known antibody sequences. Comparison of the protein sequence (precursor polypeptide) containing the leader sequence, as deduced from the cloned RNA sequence, with a database containing known human antibody protein sequences (such as the Vbase as described on page 71, lines 7-8 of the application) will reveal which part of the protein is the signal peptide and where the mature protein starts. At the earliest priority date of the application, several of these databases were available, including the above Vbase as well as the Kabat [1] or IMGT [2] databases.

9. More particularly, these databases contain collections of all germline signal peptides of all human antibodies, and a simple alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with these signal peptide databases would reveal which part of the sequences are the signal peptides and which part of the sequences are the V_H/V_L sequences. Moreover, comparison/alignment of the derived amino acid sequences (V_H/V_L sequences plus leader sequences) with the collection of mature human V_H and V_L sequences in these databases would reveal where the mature proteins

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start.

As an example, in Figure 1, a selection of V_H germline leader peptides is shown (screenshot from the Vbase). The leader peptide of V_H 7D8, MELGLSWIFLLAILKGVQC, is easily identified as sequence VH3 3-09.

Alternatively, using the DNA plot module on the Vbase site (which, to my knowledge, was available prior to the earliest priority date of 17 October 2002) it is possible to align the nucleotide sequence of your rearranged V gene to its closest mature germline V. In Figure 2 this was done for the V_L region of 11B8 and, as is shown in the screenshot, the start of the mature V_L encoding sequence is gaaatt, confirming the start of the mature V_L polypeptide sequence at the corresponding amino acids EI. This can also be done with the Vquest module at the IMGT site, see Figure 3.

10. The leader sequence for a V_H sequence is typically 19-21 amino acids long, and the leader sequence for a V_L kappa sequence is typically 19-23 amino acids long. A protein always starts with a Met residue, so the leader sequences always start with a Met residue. A V_H sequence may start with a Glu or Gln residue, a V_L kappa sequence may start with Ala, Asp, Val, Asn or Glu. V_H signal peptides can end with Cys, Ala, Ser or Pro, and V_L kappa signal peptides can end with Cys, Ala, Gly or Glu. Accordingly, a person skilled in the art would know from studying the nucleotide and amino acid sequences (SEQ ID Nos: 1-12) that the leader sequences are indeed included. Also a person skilled in the art would note that the amino acid sequences are longer than usual for the mature amino acid sequences. This information was known to a person skilled in the art at the earliest priority date of the application and further supports that the leader sequences are indeed included in the variable heavy chain and light chain amino acid sequences as identified by SEQ ID Nos: 2, 6, 10, 4, 8 and 12, respectively.

11. Alternatively, at the earliest priority date of the application several predictive methods were described, such as [3-5], by which signal peptide sequences could be predicted from a protein sequence. Using the SignalP server (which was available prior to the earliest priority date of 17 October 2002) we have performed this for the V_H polypeptide of antibody 7D8 and a screenshot of the result is shown in Figure 4, indicating the signal peptide cleavage site between the C and E amino acid, confirming the start of the mature V_H region as EV.

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12. In conclusion, it is clear that the claims defining the mature antibodies by the variable heavy chain and light chain amino acid sequences by mistake contain the leader sequences and that nothing else was intended than to define the mature antibodies without these leader sequences.

13. 2F2, 7D8 and 11B8 are characterized by having the following V_H CDR1 regions in the application, cf. for example claims 33, 36 and 40 of the application:

	2F2	**7D8**	**11B8**
V_H CDR1	SEQ ID NO:13	SEQ ID NO:19	SEQ ID NO:25

The CDR regions (SEQ ID Nos: 13-18, 19-24, 25-30) are highlighted in the variable heavy and light chain sequences in Appendix B attached hereto.

14. I have been asked whether it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 (i) that the V_H CDR1 regions erroneously contain an additional amino acid, and (ii) in the affirmative, how the correct V_H CDR1 regions should read.

15. I believe that it would have been obvious for a person skilled in the art at the earliest priority date on 17 October 2002 that (ii) the V_H CDR1 regions contain an additional amino acid, and (ii) how the correct CDR1 regions should read for the below reasons.

16. At the earliest priority date of the application several different methods were available to determine the CDR regions of antibodies. The most common method was the Kabat method [1] (see also [6] page 432-433 for an comprehensive manual for assigning the CDR regions of an antibody using the Kabat numbering scheme). Analysis of SEQ ID Nos. 14, 15, 16, 17, and 18 (2F2), 20, 21, 22, 23, and 24 (7D8) and 26, 27, 28, 29, 30 (11B8) defining the V_H CDR2 and CDR3 regions and the V_L CDR1, CDR2 and CDR3 regions of 2F2, 7D8 and 11B8, respectively, shows that the Kabat numbering scheme has indeed been used in this application.

17. When applying the Kabat numbering rules to the V_H CDR1, cf. [6] page 432: *"Start; Approximately residue 31 (always 9 after a C (Cys))*

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Residues before; always CXXXXXXXX (X meaning; any amino acid)
Residue after; Always W. Typically WV, but also WI, WA
Length; 5-7 residues "

It is obvious when applying these rules to the V_H CDR1 regions that the respective V_H CDR1 regions should be "Asp Tyr Ala Met His" for SEQ ID No 13, "Asp Tyr Ala Met His" for SEQ ID No 19 and "Tyr His Ala Met His" for SEQ ID No 25, respectively. When comparing these to the V_H CDR1 sequences as defined in the application, it appears that, by mistake, an extra amino acid has been added to these V_H CDR1 regions.

18. In conclusion, in view of the Kabat rules consistently applied in the application to designate the CDR regions, it is clear that the addition of a further amino acid to the V_H CDR1 sequences was a mistake and that nothing else was intended than to define the V_H CDR1 sequences without inclusion of this additional amino acid.

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

6 Aug 2009
Date

Tom Vink

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References

1. Kabat, E.A., et al., *Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242.* Bethesda, MD: US Department of Health and Human Services, 1991.
2. Lefranc, M.P., *IMGT, the international ImMunoGeneTics database.* Nucleic Acids Res, 2001. **29**(1): p. 207-9.
3. Chou, K.C., *Prediction of signal peptides using scaled window.* Peptides, 2001. **22**(12): p. 1973-9.
4. Chou, K.C., *Using subsite coupling to predict signal peptides.* Protein Eng, 2001. **14**(2): p. 75-9.
5. Nielsen, H., et al., *Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites.* Protein Eng, 1997. **10**(1): p. 1-6.
6. Kontermann, R. and S. Dübel, *Antibody engineering.* 2001: Springer Heidelberg.

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Apendix A

<210> 1 V_H 2F2
<211> 424
<212> DNA
<213> Homo sapiens

atggagttgg gactgagctg gattttcctt ttggctattt taaaaggtgt ccagtgtgaa 60
gtgcagctgg tggagtctgg gggaggcttg gtacagcctg gcaggtccct gagactctcc 120
tgtgcagcct ctggattcac ctttaatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggttccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaagtc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag 424

<210> 2 V_H 2F2
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

Met	Glu	Leu	Gly	Leu	Ser	Trp	Ile	Phe	Leu	Leu	Ala	Ile	Leu	Lys	Gly
1				5				10					15		
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Asn	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Ser	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

<210> 3 V_L 2F2
<211> 382
<212> DNA
<213> Homo sapiens

<400> 3
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gaaattgtgt tgacacagtc tccagccacc ctgtctttgt ctccagggga aagagccacc 120
ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240

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aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac 382

<210> 4 V_L 2F2
<211> 127
<212> PRT
<213> Homo sapiens

<400> 4

Met	Glu	Ala	Pro	Ala	Gln	Leu	Leu	Phe	Leu	Leu	Leu	Leu	Trp	Leu	Pro
1				5				10						15	
Asp	Thr	Thr	Gly	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser
			20					25					30		
Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser
		35					40					45			
Val	Ser	Ser	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro
	50					55					60				
Arg	Leu	Leu	Ile	Tyr	Asp	Ala	Ser	Asn	Arg	Ala	Thr	Gly	Ile	Pro	Ala
65					70					75					80
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser
				85					90					95	
Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys	Gln	Gln	Arg	Ser
			100					105					110		
Asn	Trp	Pro	Ile	Thr	Phe	Gly	Gln	Gly	Thr	Arg	Leu	Glu	Ile	Lys	
		115					120					125			

<210> 5 V_H 7D8
<211> 424
<212> DNA
<213> Homo sapiens

<400> 5
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tgtgcagcct ctggattcac ctttcatgat tatgccatgc actgggtccg gcaagctcca 180
gggaagggcc tggagtgggt ctcaactatt agttggaata gtggtaccat aggctatgcg 240
gactctgtga agggccgatt caccatctcc agagacaacg ccaagaactc cctgtatctg 300
caaatgaaca gtctgagagc tgaggacacg gccttgtatt actgtgcaaa agatatacag 360
tacggcaact actactacgg tatggacgtc tggggccaag ggaccacggt caccgtctcc 420
tcag 424

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<210> 6 V_H 7D8
<211> 141
<212> PRT
<213> Homo sapiens

<400> 6

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Met Glu Leu Gly Leu Ser Trp Ile Phe Leu Leu Ala Ile Leu Lys Gly
 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
            20                  25                  30
Pro Asp Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45
His Asp Tyr Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Thr Ile Ser Trp Asn Ser Gly Thr Ile Gly Tyr Ala
65                  70                  75                  80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Leu
            100                 105                 110
Tyr Tyr Cys Ala Lys Asp Ile Gln Tyr Gly Asn Tyr Tyr Tyr Gly Met
        115                 120                 125
Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
```

<210> 7 V_L 7D8
<211> 382
<212> DNA
<213> Homo sapiens

<400> 7

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gaaattgtgt tgacacagtc tccagccacc ctgtctttgt ctccagggga aagagccacc 120
ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcaact ggccgatcac cttcggccaa 360
gggacacgac tggagattaa ac                                          382
```


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<210> 8 V_L 7D8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 8

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 1               5                  10                  15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asn Trp Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
        115                 120                 125
```

<210> 9 V_H 11B8
<211> 433
<212> DNA
<213> Homo sapiens

<400> 9

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gttcagctgg tgcagtctgg gggaggcttg gtacatcctg gggggtccct gagactctcc 120
tgtacaggct ctggattcac cttcagttac catgctatgc attgggttcg ccaggctcca 180
ggaaaaggtc tggaatgggt atcaattatt gggactggtg gtgtcacata ctatgcagac 240
tccgtgaagg gccgattcac catctccaga gacaatgtca agaactcctt gtatcttcaa 300
atgaacagcc tgagagccga ggacatggct gtgtattact gtgcaagaga ttactatggt 360
gcggggagtt tttatgacgg cctctacggt atggacgtct ggggccaagg gaccacggtc 420
accgtctcct cag                                                    433
```


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<210> 10 V_H 11B8
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

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 1               5                  10                  15
Val Gln Cys Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val His
            20                  25                  30
Pro Gly Gly Ser Leu Arg Leu Ser Cys Thr Gly Ser Gly Phe Thr Phe
        35                  40                  45
Ser Tyr His Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
Glu Trp Val Ser Ile Ile Gly Thr Gly Gly Val Thr Tyr Tyr Ala Asp
65                  70                  75                  80
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Val Lys Asn Ser
                85                  90                  95
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Met Ala Val Tyr
            100                 105                 110
Tyr Cys Ala Arg Asp Tyr Tyr Gly Ala Gly Ser Phe Tyr Asp Gly Leu
        115                 120                 125
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135                 140
```

<210> 11 V_L 11B8
<211> 382
<212> DNA
<213> Homo sapiens

<400> 11

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gaaattgtgt tgacacagtc tccagccacc ctgtctttgt ctccagggga aagagccacc 120
ctctcctgca gggccagtca gagtgttagc agctacttag cctggtacca acagaaacct 180
ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 240
aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 300
gaagattttg cagtttatta ctgtcagcag cgtagcgact ggccgctcac tttcggcgga 360
gggaccaagg tggagatcaa ac                                          382
```


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<210> 12 V_L 11B8
<211> 127
<212> PRT
<213> Homo sapiens

<400> 12

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Met Glu Ala Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
 1               5                  10                  15
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
            20                  25                  30
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45
Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
    50                  55                  60
Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala
65                  70                  75                  80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                85                  90                  95
Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser
            100                 105                 110
Asp Trp Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125
```


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Appendix B

<210> 2 V_H 2F2 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 2

Met	Glu	Leu	Gly	Leu	Ser	Trp	Ile	Phe	Leu	Leu	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	[illegible]	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Asn	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Ser	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

<210> 6 V_H 7D8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 141
<212> PRT
<213> Homo sapiens

<400> 6

Met	Glu	Leu	Gly	Leu	Ser	Trp	Ile	Phe	Leu	Leu	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln
			20					25					30		
Pro	Asp	Arg	Ser	Leu	Arg	Leu	Ser	[illegible]	Ala	Ala	Ser	Gly	Phe	Thr	Phe
		35					40					45			
His	Asp	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Thr	Ile	Ser	Trp	Asn	Ser	Gly	Thr	Ile	Gly	Tyr	Ala
65					70					75					80
Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn
				85					90					95	
Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Leu
			100					105					110		
Tyr	Tyr	Cys	Ala	Lys	Asp	Ile	Gln	Tyr	Gly	Asn	Tyr	Tyr	Tyr	Gly	Met
		115					120					125			
Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser			
	130					135					140				

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<210> 10 V_H 11B8 (Additional amino acid in V_H CDR1 region has been underlined.)
<211> 144
<212> PRT
<213> Homo sapiens

<400> 10

Met	Glu	Leu	Gly	Leu	Ser	Trp	Val	Phe	Leu	Val	Ala	Ile	Leu	Lys	Gly
1				5					10					15	
Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Leu	Val	His
			20					25					30		
Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Xaa	Thr	Gly	Ser	Gly	Phe	Thr	Phe
		35					40					45			
Ser	Tyr	His	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
	50					55					60				
Glu	Trp	Val	Ser	Ile	Ile	Gly	Thr	Gly	Gly	Val	Thr	Tyr	Tyr	Ala	Asp
65					70					75					80
Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Val	Lys	Asn	Ser
				85					90					95	
Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Met	Ala	Val	Tyr
			100					105					110		
Tyr	Cys	Ala	Arg	Asp	Tyr	Tyr	Gly	Ala	Gly	Ser	Phe	Tyr	Asp	Gly	Leu
		115					120					125			
Tyr	Gly	Met	Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser
	130					135					140				

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Figure 1
A screenshot is shown of part of the germline V_H signal peptides in the Vbase database.

VH Leader - Amino acid sequence alignment

		-19 -10 -1
		| | |
VH1	1-02	MDWIWRILFLVAAATGAHS
	1-03	MDWIWRILFLVAAATGAHS
	1-08	MDWIWRILFLVAAATSAHS
	1-18	MDWIWSILFLVAAPTGAHS
	1-24	MDCTWRILFLVAAATGTHA
	1-45	MDWIWRILFLVAAATDAYS
	1-46	MDWIWRVFCLLAVAPGAHS
	1-58	MDWIWRILFLVGAATGAHS
VH2	2-05	MDTLCSTLLLLTIPSWVLS
	2-26	MDTLCYTLLLLTTPSWVLS
VH3	3-07	MELGLSWVFLVAILEGVQC
	3-09	MELGLSWIFLLAILKGVQC
	3-11	MEFGLSWVFLVAIIKGVQC
	3-13	MELGLSWVFLVAILEGVQC
	3-15	MEFGLSWIFLAAILKGVQC
	3-20	MEFGLSWVFLVAILKGVQC
	3-21	MELGLRWVFLVAILEGVQC
	3-23	MEFGLSWLFLVAILKGVQC
	3-30	MEFGLSWVFLVALLRGVQC
	3-33	MEFGLSWVFLVALLRGVQC

Figure 2
A screenshot of an analysis performed using the DNAplot module on the Vbase site. The complete DNA sequence of the V_L 11B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

Thank you for using DNAPLOT and V BASE !

Alignment for V segment

EMBL	Locus	Name	score	GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGG
X01668	L6	Vg/38K...+	1305	.....
X17264	L20	Vg^n/13K11...+	1278	.....
L19271	-	3A7	1260	.....
L19272	-	3A9	1242	.....G.....
X93639	A27	DPK22/A27...+	1224	.....G.....G.....

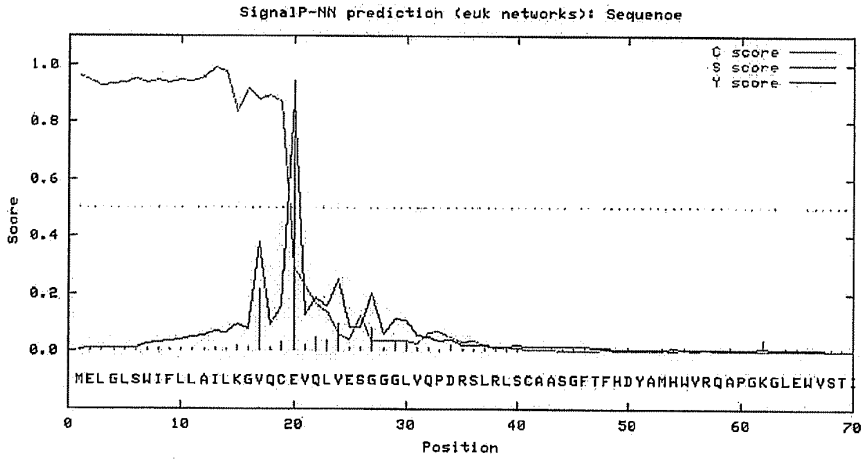
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Figure 3
A screenshot of an analysis performed using the VQUEST module on the IMGT site. The complete DNA sequence of the V_L !1B8 antibody was analyzed and an alignment is shown of this sequence with the germline mature V_L regions in the database.

```
Using neural networks (NN) and hidden Markov models (HMM) trained on eukaryotes


>Sequence

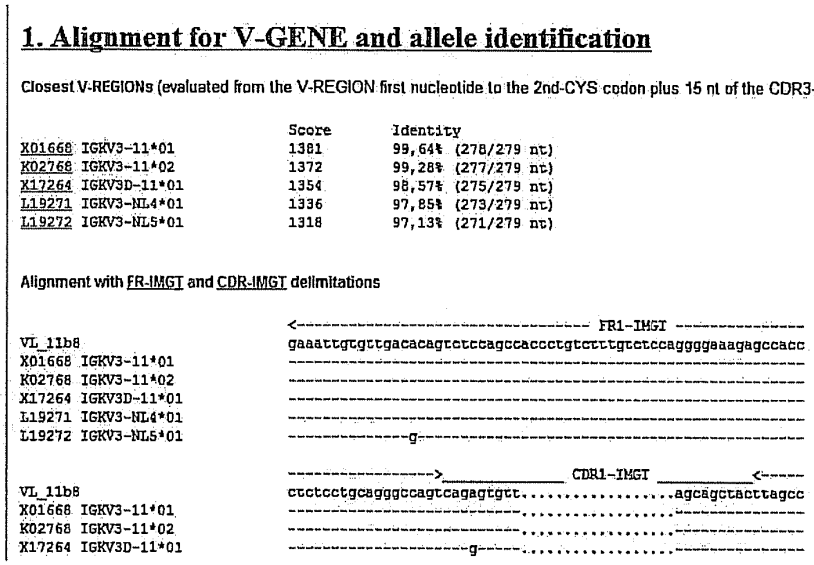

SignalP-NN result:
```




```
# data

>Sequence                 length = 70
# Measure  Position  Value  Cutoff  signal peptide?
  max. C     20       0.940   0.32   YES
  max. Y     20       0.883   0.33   YES
  max. S     13       0.988   0.87   YES
  mean S      1-19    0.930   0.48   YES
       D      1-19    0.907   0.43   YES
# Most likely cleavage site between pos. 19 and 20: VQC-EV
```

Figure 4
The V_H polypeptide sequence of antibody 7D8 was analyzed using the SignalP server. A screenshot of the resulting prediction of the signal peptide cleavage site is shown.



1. Alignment for V-GENE and allele identification

Closest V-REGIONs (evaluated from the V-REGION first nucleotide to the 2nd-CYS codon plus 15 nt of the CDR3-IMGT)

	Score	Identity
X01668 IGKV3-11*01	1381	99,64% (278/279 nt)
K02768 IGKV3-11*02	1372	99,28% (277/279 nt)
X17264 IGKV3D-11*01	1354	98,57% (275/279 nt)
L19271 IGKV3-NL4*01	1336	97,85% (273/279 nt)
L19272 IGKV3-NL5*01	1318	97,13% (271/279 nt)

Alignment with FR-IMGT and CDR-IMGT delimitations

```
                          <---------------------------------- FR1-IMGT ----------------
VL_11b8                   gaaattgtgttgacacagtctccagccaccctgtctttgtctccaggggaaagagccacc
X01668 IGKV3-11*01        ---------------------------------------------------------
K02768 IGKV3-11*02        ---------------------------------------------------------
X17264 IGKV3D-11*01       ---------------------------------------------------------
L19271 IGKV3-NL4*01       ---------------------------------------------------------
L19272 IGKV3-NL5*01       ----------g----------------------------------------------

                          ----------------->_____________ CDR1-IMGT _________<-----
VL_11b8                   ctctcctgcagggccagtcagagtgtt.................agcagctacttagcc
X01668 IGKV3-11*01        --------------------------.................---------------
K02768 IGKV3-11*02        --------------------------.................---------------
X17264 IGKV3D-11*01       --------------------g-----.................---------------
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(54) ANTI-TIM-3 ANTIBODY

(57) The present invention provides an anti-human TIM-3 antibody which binds to the amino acid sequence of the extracellular region of TIM-3 or its three-dimensional structure thereof and exhibits higher effector activity such as an antibody-dependent cellular cytotoxicity (ADCC activity) for diseases relating to a human TIM-3 expressing cell. The present invention provides a monoclonal antibody or antibody fragment thereof which binds to the amino acid sequence of the extracellular region of TIM-3 or its three-dimensional structure and exhibits ADCC activity; a hybridoma which produces the antibody; a DNA encoding the antibody; a vector comprising the DNA; a transformant which is obtainable by introducing the vector; a method for producing the antibody or the antibody fragment thereof which comprises using the hybridoma or the transformant; a therapeutic agent and a diagnostic agent comprising the antibody or the antibody fragment thereof as an active ingredient. In addition, the present invention provides an anti-human TIM-3 antibody having high ADCC activity by screening an anti-human TIM-3 antibody which competes with the monoclonal antibody or the antibody fragment thereof.

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Description

Technical Field

[0001] The present invention relates to a monoclonal antibody or an antibody fragment thereof, which binds to the extracellular region of T-cell immunoglobulin and mucin domain containing molecule-3 (hereinafter referred to as "TIM-3") and exhibits antibody-dependent cellular cytotoxicity (hereinafter referred to as "ADCC"); a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof.

Background Art

[0002] TIM-3 gene family consists of eight genes in mouse and three genes in human, and each of these genes are located at chromosome 11 and at chromosome 5q33 respectively (Non-patent Document 1). These gene regions are known to be related with autoimmune diseases and allergic diseases. TIM protein is a type I transmembrane protein having a structurally conserved immunoglobulin variable (IgV) domain and a mucin domain.

[0003] TIM protein was considered to be specifically expressed on T cells and directly regulate the T cell activity, but there are recent reports on expression of TIM-3 protein in antigen-presenting cells and on their functions (Non-patent Document 2). According to the crystal structure analysis, the TIM protein has a conserved protein structure and has a ligand binding site in the IgV domain.

[0004] TIM-3 was identified as a molecule specifically expressed on mouse Th1 cells but not on Th2 cells (Non-patent Document 3). The DNA sequence, the amino acid sequence and the three-dimensional structure of TIM-3 is available in the public data base such as the GenBank accession number NM_032782 and NM_134250. TIM-3 is also known as HAVCR2.

[0005] In humans, as similar to mice, TIM-3 is expressed on T-cells as well as phagocytic cells such as macrophages and dendritic cells. Binding of TIM-3 to a protein ligand (e.g., galectin-9) can inhibit the Th1 response via mechanism of apoptosis induction, and therefore lead to such as induction of peripheral tolerance.

[0006] The reduction in expression of human TIM-3 with siRNA or the inhibition of human TIM-3 by blocking-antibody increased the secretion of interferon γ (IFN-γ) from CD4 positive T-cells, supporting the inhibitory role of TIM-3 in human T cells. In phagocytes, TIM-3 also functions as a receptor for recognizing the apoptosis cells.

[0007] Analysis of clinical samples from autoimmune disease patients showed no expression of TIM-3 in CD4 positive cells. In particular, in T cell clones derived from the cerebrospinal fluid of patients with multiple sclerosis, the expression level of TIM-3 was lower and the secretion level of IFN-γ was higher than those of clones derived from normal healthy persons (Non-patent Document 4). There are reports on relation of TIM-3 with allergic diseases or asthma (Patent Documents 1 and 2).

[0008] According to the microarray analysis of hematopoietic stem cells from acute myeloid leukemia (hereinafter referred to as "AML") patients and normal hematopoietic stem cells, TIM-3 is expressed on AML stem cells and therefore the analysis suggested involvement of TIM-3 in hematological malignancy (Non-patent Document 5 and Patent Document 3).

[0009] Examples of the anti-TIM-3 monoclonal antibodies which were established up to now include anti-human TIM-3 rat monoclonal antibody (Clone 344823, manufactured by R&D Systems) and anti-human TIM-3 mouse monoclonal antibody (Clone F38-2E2, manufactured by R&D Systems).

Related Art

Pantent Literature

[0010]

Pantent Literature 1 WO96/27603
Pantent Literature 2 WO2003/063792
Pantent Literature 3 WO2009/091547

Non-Pantent Literature

[0011]

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Non-Pantent Literature 1 Hafler DA et al., JExp Med. 205: 2699-701 (2008)
Non-Pantent Literature 2 Anderson AC et al., Science 318: 1141-3 (2007)
Non-Pantent Literature 3 Monney L et al., Nature 415: 536-41 (2002)
Non-Pantent Literature 4 Koguchi K et al., J Exp Med. 203: 1413-8 (2006)
Non-Pantent Literature 5 Majeti R et al., Proc Natl Acad Sci USA 2009 Mar 3; 106 (9): 3396-401.

Disclosure of Invention

Problems to Be Solved by the Invention

[0012] However, there is no report on monoclonal antibody against human TIM-3 having ADCC activity. Therefore, the object of the present invention is to provide a monoclonal antibody or an antibody fragment which binds to the amino acid sequence or the three-dimensional structure of the extracellular region of TIM-3 and expresses ADCC activity. In addition, the object of the present invention is to provide an anti-human TIM-3 antibody having high ADCC activity by screening an anti-human TIM-3 antibody which competes with the monoclonal antibody or the antibody fragment thereof.
[0013] In addition, the present invention is to provide a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof as an active ingredient.

Means for Solving the Problems

[0014] The gist of the present invention relates to the followings.

(1) A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (i) to (iii):

(i) an antibody which comprises a heavy chain (hereinafter referred to as "H chain") of an antibody which comprises complementary determining regions (hereinafter referred to as "CDR") 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises a light chain (hereinafter referred to as "L chain") of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) an antibody which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) an antibody which comprises an H chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

(2) The monoclonal antibody or the antibody fragment thereof described in the above (1), wherein the monoclonal antibody binds to the same epitope to which one antibody selected from the above (i) to (iii) binds.
(3) A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (a) and (b):

(a) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

(4) The monoclonal antibody or the antibody fragment thereof described in the above (3), which binds to the same epitope to which one antibody selected from the above (a) or (b) binds.
(5) The monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (4), which is a recombinant antibody.
(6) The monoclonal antibody or the antibody fragment thereof described in the above (5), which is a recombinant antibody selected from a human chimeric antibody, a humanized antibody and a human antibody.
(7) A monoclonal antibody or an antibody fragment thereof, which is one selected from the following (i) to (iii):

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(i) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

(8) A monoclonal antibody and an antibody fragment thereof, which is one selected from the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

(9) A monoclonal antibody and an antibody fragment thereof which binds to the amino acid sequences at positions 67 to 105, the amino acid sequences at positions 67 to 96 or the amino acid sequences at positions 67 to 87 in the amino acid sequence of IgV domain of human TIM-3 represented by SEQ ID NO: 53.
(10) The antibody fragment described in any one of the above (1) to (9), wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv) and a peptide comprising CDR.
(11) A DNA which encodes the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(12) A recombinant vector which comprises the DNA described in the above (11).
(13) A transformant obtainable by introducing the recombinant vector described in the above (12) into a host cell.
(14) A process for producing the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10), which comprises culturing the transformant described in the above (13) in culture to form and accumulate the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10), and recovering the monoclonal antibody or the antibody fragment thereof from the culture.
(15) A method for immunologically detecting or measuring a human TIM-3 which comprises using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(16) A reagent for detecting or measuring a human TIM-3 which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(17) A diagnostic agent for a disease relating to a human TIM-3 positive cell which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(18) A method for diagnosing a disease relating to a human TIM-3 positive cell which comprises detect or measure a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(19) A method for diagnosing a disease relating to a human TIM-3 positive cell, which comprises ditecting or measuring a human TIM-3 using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(20) Use of the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10) for the manufacture of a diagnostic agent for a disease relating to a human TIM-3 positive cell.
(21) A therapeutic agent for a disease relating to a human TIM-3 positive cell, which comprises the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(22) A therapeutic method for a disease relating to a human TIM-3 positive cell, which comprises inducing cell death of a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10).
(23) Use of the monoclonal antibody or the antibody fragment thereof described in any one of the above (1) to (10) for the manufacture of a therapeutic agent for a disease relating to a human TIM-3 positive cell.

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Effects of the Invention

[0015] The monoclonal antibody or the antibody fragment of the present invention specifically recognizes the amino acid sequence or the three-dimensional structure of the extracellular region of human TIM-3, and binds to the extracellular region. The amino acid sequence or the three-dimensional structure of the extracellular region of human TIM-3 which is recognized by the monoclonal antibody or the antibody fragment of the present invention is different from those recognized by known anti-TIM-3 monoclonal antibodies. Therefore, the monoclonal antibody or the antibody fragment of the present invention has higher ADCC activity. The monoclonal antibody or the antibody fragment of the present invention which specifically binds to the extracellular region of human TIM-3 and has higher ADCC activity is useful as a therapeutic agent and a diagnostic agent for a disease relating to a human TIM-3 positive cell.

[0016] The present invention may provide a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof.

Brief Description of the Drawings

[0017]

[Fig. 1] Fig. 1 is the amino acid sequences which represent HV0, HV3, HV4, HV5, HV6, HV7, HV8, HV10, and HV12 of the designed H chain variable regions of antibody 8213, respectively.

[Fig. 2] Fig. 2 is the amino acid sequences which represent LV0, LV2, LV4, LV5, LV6, LV7, and LV9 of the designed L chain variable regions of antibody 8213.

Embodiments for Carrying Out the Invention

[0018] The human TIM-3 of the present invention include a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782, and having a function of TIM-3; a polypeptide comprising an amino acid sequence having at least 60% homology, preferably at least 80% homology, more preferably at least 90% homology, and most preferably at least 95% homology, with the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782, and having a function of TIM-3; and the like.

[0019] The polypeptide comprising an amino acid sequence wherein one or more amino acid residue(s) is/are deleted, substituted and/or added in the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be obtained, for example, by introducing a site-directed mutation into DNA encoding the polypeptide comprising the amino acid sequence represented by SEQ ID NO: 53 by site-directed mutagenesis described in Molecular Cloning, A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory Press, 1989), Current Protocols in Molecular Biology (John Wiley & Sons, 1987-1997), Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci. USA, 79, 6409 (1982), Gene, 34, 315 (1985), Proc. Natl. Acad. Sci. USA, 82, 488 (1985), or the like.

[0020] The number of amino acid residues which are deleted, substituted or added is not particularly limited, and the number is preferably, 1 to dozens, such as 1 to 20, and more preferably 1 to several, such as 1 to 5.

[0021] As a gene which encodes human TIM-3, examples include the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782. Examples also include a gene comprising a nucleotide sequence in which at least one nucleotide is deleted, substituted or added in the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 and comprising a DNA encoding a polypeptide having a function of TIM-3; a gene comprising an nucleotide sequence having at least 60% homology, preferably at least 80% homology, and more preferably at least 95% homology, with the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 and comprising a DNA encoding a polypeptide having a function of TIM-3; a gene which hybridizes with the DNA consisting of the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 under stringent conditions and encodes a polypeptide having a function of TIM-3; and the like.

[0022] In the present invention, the DNA which hybridizes under stringent conditions refers to a DNA which is obtained by colony hybridization, plaque hybridization, Southern blot hybridization, DNA microarray analysis, or the like using a DNA consisting of the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782 as a probe.

[0023] A specific example of such DNA is a DNA which can be identified by performing hybridization at 65°C in the presence of 0.7 to 1.0 mol/L sodium chloride using a filter or a slide glass with colony- or plaque-derived DNA, PCR products or oligo DNA encoding the DNA sequence immobilized thereon, and then washing the filter or a slide glass at 65°C with a 0.1 to 2-fold concentration SSC solution (1-fold concentration SSC solution: 150 mmol/L sodium chloride and 15 mmol/L sodium citrate). Hybridization can be carried out according to the methods described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular

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Biology, John Wiley & Sons (1987-1997); DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995); and the like.

[0024] Specifically, the DNA capable of hybridization includes DNA having at least 60% or more homology, preferably 80% or more homology, and more preferably 95% or more homology to the nucleotide sequence represented by SEQ ID NO: 52 or NCBI Accession No. NM_032782.

[0025] In the nucleotide sequence of the gene encoding a protein of a eukaryote, genetic polymorphism is often recognized. The TIM-3 gene used in the present invention also includes a gene in which small modification is generated in the nucleotide sequence by such polymorphism.

[0026] The number of the homology described in the present invention may be a number calculated by using a homology search program known by the skilled person, unless otherwise indicated. Regarding the nucleotide sequence, the number may be calculated by using a default parameter in BLAST [J. Mol. Biol., 215, 403 (1990)] or the like, and regarding the amino acid sequence, the number may be calculated by using a default parameter in BLAST2 [Nucleic Acids Res., 25, 3389 (1997); Genome Res., 7, 649 (1997); http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html] or the like.

[0027] As the default parameter, G (cost to open gap) is 5 for the nucleotide sequence and 11 for the amino acid sequence; -E (cost to extend gap) is 2 for the nucleotide sequence and 1 for the amino acid sequence; -q (penalty for nucleotide mismatch) is -3; -r (reward for nucleotide match) is 1; -e (expect value) is 10; -W (wordsize) is 11 residues for the nucleotide sequence and 3 residues for the amino acid sequence;.-y (Dropoff (X) for blast extensions in bits) is 20 for blastn and 7 for a program other than blastn; -X (X dropoff value for gapped alignment in bits) is 15; and Z (final X dropoff value for gapped alignment in bits) is 50 for blastn and 25 for a program other than blastn (http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html).

[0028] The polypeptide comprising a partial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be prepared according to a method known by the skilled person. For example, it can be prepared by deleting a part of DNA encoding the amino acid sequence represented by SEQ ID NO: 52 and culturing a transformant into which an expression vector containing the DNA is introduced.

[0029] Also, based on the thus prepared polypeptide or DNA, a polypeptide comprising an amino acid sequence in which one or more amino acid(s) is/are deleted, substituted or added in a partial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can be prepared in the same manner as described above.

[0030] The polypeptide comprising a parcial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782; or the polypeptide comprising an amino acid sequence in which at least one amino acid is deleted, substituted or added in a parcial sequence of the amino acid sequence represented by SEQ ID NO: 53 or NCBI Accession No. NM_032782 can also be produced by a chemical synthesis method such as fluorenylmethoxycarbonyl (Fmoc) method or t-butyloxycarbonyl (tBoc) method.

[0031] The extracellular region of human TIM-3 of the present invention includes, for example, regions predicted by using the amino acid sequence of the polypeptide represented by SEQ ID NO: 53 with conventionally known transmembrane region prediction program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui submit.html), TMHMM ver. 2 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), ExPASy Proteomics Server (http://Ca.expasy.org/) or SMART (http://smart.embl-heidelberg.de/).

[0032] In the present invention, the amino acid sequence of extracellular region of human TIM-3 includes the amino acid sequence at positions 1 to 201 in the amino acid sequence represented by SEQ ID NO: 53, which is the region of the extracellular region predicted by SMART.

[0033] In the present invention, the three-dimensional structure of the extracellular region of human TIM-3 is not limited, so long as the extracellular region of human TIM-3 comprising the amino acid sequence represented by SEQ ID NO: 53 or GenBank accession number NM_032782 has the same structure as in the natural state. The three-dimensional structure of the extracellular region of human TIM-3 in the natural state is a natural type of the three-dimensional structure of human TIM-3 expressed on the surface of the cell membrane.

[0034] Regarding the function of human TIM-3, binding of TIM-3 with a protein ligand (e.g., galectin 9) can inhibit the Th1 response thorough mechanism such as apoptosis induction in Th1 cells, therefore leading to induction of peripheral tolerance. In phagocytes, human TIM-3 functions as a receptor for recognizing the apoptosis cells.

[0035] Binding of the antibody or antibody fragment thereof in the present invention to an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof can be confirmed by a conventionally known immunological immunological detection method using human TIM-3 expressing cells such as a radioimmuno assay with a solid phase sandwich method or enzyme-linked immunosorbent assay (ELISA), and preferably a fluorescent cell staining method in which the binding ability of a cell expressing a specified antigen and an antibody for the specific antigen is confirmed.

[0036] Specific examples include a fluorescent antibody staining method using an FMAT8100HTS System (manufactured by Applied Biosystems) [Cancer Immunol. Immunother, 36, 373 (1993)] or the like, a fluorescent cell staining

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method using flow cytometry, surface plasmon resonance using a Biacore System (manufactured by GE Healthcare) or the like, and the like.

[0037] Furthermore, a known immunological detection methods [Monoclonal Antibodies - Principles and practice, Third edition, Academic Press (1996), Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988), Monoclonal Antibody Experimental Manual, Kodan-sha Scientific (1987)] and the like can be combined to confirm the binding.

[0038] In the present invention, the cell expressing human TIM-3 may be any cell, so long as it expresses human TIM-3, and examples include a cell which is naturally present in the human body, a cell line established from the cell which is naturally present in the human body, a cell obtained by a recombinant technique and the like.

[0039] Example of the cell which is naturally present in the human body includes a cell expressing the TIM-3 in the body of a patient suffering from cancer, autoimmune disease or allergic disease, concretely, the cells are Th1 cells, macropharges, dendritic cells, and the like.

[0040] Example of a cell line established from the cell which is naturally present in the human body include a cell line expressing human TIM-3, among cell lines prepared by establishing the human TIM-3-expressing cells obtained from the above cancer patients, and examples include human acute myeloid leukemia cell line KG-1 (ATCC Accession No: CCL-246), human Burkitt's lymphoma cell line Daudi (ATCC Accession No: CCL-213) which are established from a human cell and the like.

[0041] Specific examples of the cell obtained by a recombinant technique may include a human TIM-3-expressing cell obtained by introducing an expression vector comprising a human TIM-3-encoding cDNA into an insect cell, an animal cell or the like, and others.

[0042] In addition, the present invention relates to a monoclonal antibody which recognizes an amino acid sequence or a three-dimensional structure of an extracellular region of human TIM-3 and exhibits ADCC activity.

[0043] The ADCC activity of the present invention is a reaction in which an antibody which is bound to human TIM-3 on the cell surface is bound to FcγRIIIa on the surface of mainly a natural killer cell (hereinafter referred to as NK cell) through Fc region and therefore a cytotoxic molecule such as molecules perforin, and granzyme released from an NK cell leads to cell lysis [Clark M, Chemical Immunology, 65, 88 (1997); Gorter A *et al.,* Immunol. Today, 20, 576 (1999)].

[0044] The antibody of the present invention may be any antibodies as long as it is an antibody or antibody fragment thereof which recognizes an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof and binds to the extracellular region; or an antibody or antibody fragment thereof which binds an extracellular region of human TIM-3 or a three-dimensional structure thereof and have ADCC activity.

[0045] Specific example of the antibody of the present invention may include an antibody which binds to the amino acid sequence selected from sequences preferably at positions 67 to 105, more preferably at positions 67 to 96, and most preferably at positions 67 to 87 among the amino acid sequence of extracellular region of human TIM-3 comprising of the sequence at positions 1 to 201 in the sequence represented by SEQ ID NO: 53, or the three-dimensional structure thereof.

[0046] The example of the antibody includes the monoclonal antibody and the antibody fragment thereof as described in the following (i) to (iii):

(i) a monoclonal antibody or an antibody fragment thereof which comprises a heavy chain (hereinafter referred to as "H chain") of an antibody which comprises complementary determining regions (hereinafter referred to as "CDR") 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises a light chain (hereinafter referred to as "L chain") of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively.

(ii) a monoclonal antibody or an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively.

(iii) a monoclonal antibody or an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

[0047] The example of the monoclonal antibody includes a monoclonal antibody and an antibody fragment thereof as described in the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,

(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the

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amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

[0048] In addition, the antibody of the present invention include a monoclonal antibody or an antibody fragment thereof which competes with the above monoclonal antibody in the binding of an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a monoclonal antibody or an antibody fragment thereof which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above monoclonal antibody binds.

[0049] In the present invention, an antibody which compete with a monoclonal antibody means an antibody which recognizes the same or a part of the same epitope (also referred to as antigen determinant) on the extracellular region of human TIM-3 as the monoclonal antibody of the present invention, and binds to the epitope. The antibody which binds to the same epitope as the monoclonal antibody of the present invention means an antibody which recognizes and binds to an amino acid sequence of human TIM-3 which the monoclonal antibody of the present invention recognizes.

[0050] The monoclonal antibody of the present invention includes an antibody produced by a hybridoma and a recombinant antibody produced by a transformant transformed with an expression vector containing a gene encoding an antibody.

[0051] The monoclonal antibody is an antibody secreted by a single clone antibody-producing cell, and recognizes only one epitope (also called antigen determinant) and has uniform amino acid sequence (primary structure).

[0052] Examples of the epitope include a single amino acid sequence, a three-dimensional structure comprising the amino acid sequence, a sugar chain-bound amino acid sequence, a three-dimensional structure comprising a sugar chain-bound amino acid sequence, and the like, recognized and bound by a monoclonal antibody.

[0053] The monoclonal antibody of the present invention binds preferably to the amino acid sequence at positions 22 to 131 of the IgV domain of human TIM-3 in the extracellular regions of human TIM-3 represented by SEQ ID NO: 53. Specifically, the amino acid sequence to which the monoclonal antibody of the present invention binds is preferably the amino acid sequence at positions 67 to 105, more preferably the amino acid sequence at positions 67 to 96, and most preferably the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0054] The epitope to which the monoclonal antibody of the present invention binds may preferably be included in the IgV domain of human TIM-3 represented by the amino acid sequence at positions 22 to 131 in the extracellular region of human TIM-3 represented by SEQ ID NO: 53. Specifically, the epitope to which the monoclonal antibody of the present invention binds is preferably included in the amino acid sequence at positions 67 to 105, more preferably included in the amino acid sequence at positions 67 to 96, and most preferably included in the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0055] The amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds may preferably include at least one amino acid selected from the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53 in the IgV domain of human TIM-3, and more preferably include at least one amino acid selected from the amino acids at position 67, position 74, position 76, position 78, position 79, position 81, position 83 and position 85.

[0056] The amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds may preferably include at least two or more continuous amino acids selected from positions 67 to 87 of the human TIM-3 IgV domain; and more preferably may include at least one amino acid selected from the amino acids at position 67, position 74, position 76, position 78, position 79, position 81, position 83 and position 85 and include at least two or more continuous amino acids selected from the amino acid sequence at positions 67 to 87 in the amino acid sequence represented by SEQ ID NO: 53.

[0057] Specifically, examples of the amino acid sequence of the epitope to which the monoclonal antibody of the present invention binds include the amino acid sequence comprising the amino acids at positions 67 to 74, the amino acid sequence comprising the amino acids at positions 67 to 76, the amino acid sequence comprising the amino acids at positions 67 to 78, the amino acid sequence comprising the amino acids at positions 67 to 79, the amino acid sequence comprising the amino acids at positions 67 to 81, the amino acid sequence comprising the amino acids at positions 67 to 83, the amino acid sequence comprising the amino acids at positions 67 to 85,

the amino acid sequence comprising the amino acids at positions 74 to 76, the amino acid sequence comprising the amino acids at positions 74 to 78, the amino acid sequence comprising the amino acids at positions 74 to 79, the amino acid sequence comprising the amino acids at positions 74 to 81, the amino acid sequence comprising the amino acids at positions 74 to 83, the amino acid sequence comprising the amino acids at positions 74 to 85,

the amino acid sequence comprising the amino acids at positions 76 to 78, the amino acid sequence comprising the amino acids at positions 76 to 79, the amino acid sequence comprising the amino acids at positions 76 to 81, the amino acid sequence comprising the amino acids at positions 76 to 83, the amino acid sequence comprising the amino acids at positions 76 to 85, the amino acid sequence comprising the amino acids at positions 78 to 79, the amino acid sequence comprising the amino acids at positions 78 to 81, the amino acid sequence comprising the amino acids at positions 78

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to 83, the amino acid sequence comprising the amino acids at positions 78 to 85,
the amino acid sequence comprising the amino acids at positions 79 to 81, the amino acid sequence comprising the amino acids at positions 79 to 83, the amino acid sequence comprising the amino acids at positions 79 to 85,
the amino acid sequence comprising the amino acids at position 81 to 83, the amino acid sequence comprising the amino acids at positions 81 to 85,
the amino acid sequence comprising the amino acids at positions 83 to 85, and the like,
in the amino acid sequence represented by SEQ ID NO: 53.

[0058] The hybridoma can be prepared, for example, by preparing the above cell expressing TIM-3 as an antigen, inducing an antibody-producing cell having antigen specificity from an animal immunized with the antigen, and fusing the antibody-producing cell with a myeloma cell. The anti-TIM-3 antibody can be obtained by culturing the hybridoma or administering the hybridoma cell into an animal to cause ascites tumor in the animal and separating and purifying the culture or the ascites.

[0059] The animal immunized with an antigen may be any animal, so long as a hybridoma can be prepared, and mouse, rat, hamster, chicken, rabbit or the like is suitably used. Also, the cell having antibody-producing activity can be obtained from such an animal, and the antibody of the present invention includes an antibody produced by a hybridoma obtained by fusion of the cell after *in vitro* immunization with a myeloma cell.

[0060] In the present invention, the recombinant antibody includes an antibody produced by gene recombination, such as a human chimeric antibody, a human CDR grafted antibody, a human antibody and an antibody fragment thereof. Among the recombinant antibodies, one having character of a common monoclonal antibody, low immunogenecity and prolonged half-life in blood is preferable as a therapeutic agent. Examples of the recombinant antibody include an antibody which is prepared by modifying the above monoclonal antibody of the present invention using a recombinant technique.

[0061] The human chimeric antibody is an antibody comprising VH and VL of an antibody of a non-human animal, and a heavy chain constant region (hereinafter referred to as CH) and a light chain constant region (hereinafter referred to as CL) of a human antibody. Specifically, the human chimeric antibody of the present invention can be produced by obtaining cDNAs encoding VH and VL from a hybridoma which produces a monoclonal antibody which specifically recognizes human TIM-3 and binds to an amino acid sequence of extracellular region or a three-dimensional structure thereof, inserting each of them into an expression vector for animal cell comprising DNAs encoding CH and CL of human antibody to thereby construct a vector for expression of human chimeric antibody, and then introducing the vector into an animal cell to express the antibody.

[0062] As the CH of the human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg"), and those belonging to the hIgG class are preferred, and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. As the CL of the human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to κ class or λ class can be used.

[0063] Specific examples of the human chimeric antibody of the present invention includes a chimeric antibody which comprises VH of the antibody comprising the amino acid sequence represented by SEQ ID NO: 28 and comprises VL of the antibody comprising the amino acid sequence represented by SEQ ID NO: 30.

[0064] In addition, the chimeric antibody of the present invention include a chimeric antibody which competes with the above monoclonal antibody in the binding of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a chimeric antibody which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above chimeric antibody binds.

[0065] The human CDR grafted antibody is also called as a humanized antibody and is an antibody in which CDR of VH and VL of a non-human animal antibody were grafted into the appropriate site of VH and VL of a human antibody. The human CDR grafted antibody of the present invention can be produced by constructing cDNAs encoding an antibody V region in which the amino acid sequences of CDRs of VH and VL of an antibody derived from a non-human animal produced by a hybridoma which produces a monoclonal antibody which specifically recognizes three-dimensional structure of TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof are grafted into frameworks (hereinafter referred to as "FR") of VH and VL of a suitable human antibody, inserting each of them into an expression vector for animal cell comprising genes encoding CH and CL of a human antibody to thereby construct a vector for expression of humanized antibody, and introducing it into an animal cell to thereby express and produce the humanized antibody.

[0066] As the CH of the human CDR grafted antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg"), and those belonging to the hIgG class are preferred, and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. As the CL of the human CDR grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to κ class or λ class can be used.

[0067] Examples of the CDR grafted antibody of the present invention include a humanized antibody in which CDRs 1 to 3 of VH of the antibody comprises the amino acid sequences represented by SEQ ID NO: 21, 22, and 23, respectively,

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and CDRs 1 to 3 ofVL of the antibody comprises the amino acid sequences represented by SEQ ID NO: 24, 25, and 26, respectively.

[0068] Specific examples of the humanized antibody include a humanized antibody comprising at least one of the following (a) VH and (b) VL:

(a) VH of the antibody comprising the amino acid sequence represented by SEQ ID NO: 67, or an amino acid sequence in which at least one amino acid residue selected from Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 is substituted with other amino acid residue(s),

(b) VL of the antibody comprising the amino acid sequence represented by SEQ ID NO: 69, or an amino acid sequence in which at least one amino acid residue selected from Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 is substituted with other amino acid residue(s).

[0069] As the VH comprised in the humanized antibody, the following (1) to (8) are preferable:

(1) VH comprising an amino acid sequence in which Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(2) VH comprising an amino acid sequence in which Lys at position 12, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(3) VH comprising an amino acid sequence in which Val at position 20, Arg at position 38, Met at position 48, Val at position 68, Ile at position 70, Ala at position 72, Arg at position 98 and Val at position 113 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(4) VH comprising an amino acid sequence in which Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(5) VH comprising an amino acid sequence in which Lys at position 12, Arg at position 67, Val at position 68, Ala at position 72, Thr at position 74 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues;

(6) VH comprising an amino acid sequence in which Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues,

(7) VH comprising an amino acid sequence in which Arg at position 38, Met at position 48, Arg at position 67 and Thr at position 74 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues; and

(8) VH comprising an amino acid sequence in which Arg at position 38, Met at position 48 and Arg at position 98 in the amino acid sequence represented by SEQ ID NO: 67 are substituted with other amino acid residues.

[0070] The amino acid sequence of the above VH obtained by the amino acid modifications includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly or Val at position 113 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0071] Specific examples of the amino acid sequence of VH in which twelve modifications are introduced include an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0072] Examples of the amino acid sequence of VH in which eleven modifications are introduced include the following amino acid sequence (1) to (12):

(1) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at

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position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(3) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(5) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(6) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(7) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(8) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(9) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(10) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(11) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(12) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0073] Specific examples of the amino acid sequence of VH in which ten modifications are introduced include the following amino acid sequence (1) to (8):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced

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in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(8) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0074] Examples of the amino acid sequence of VH in which eight modifications are introduced include the following amino acid sequence (1) to (13):

(1) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Arg at

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position 98 with Gly and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(11) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(12) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(13) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0075] Specific examples of the amino acid sequence of VH in which seven modifications are introduced include the following amino acid sequence (1) to (11):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(2) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(6) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

(10) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and

(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0076] Specific Examples of the amino acid sequence of VH in which six modifications are introduced include the following amino acid sequence (1) to (16):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(12) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(13) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(14) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(15) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(16) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0077] Specific examples of the amino acid sequence of VH in which five modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions ofArg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ile at position 70 with Leu and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Ala at position 72 with Val and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys, Thr at position 74 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0078] Specific examples of the amino acid sequence of VH in which four modifications are introduced include the following amino acid sequence (1) to (8):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile, Arg at position 67 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0079] Specific examples of the amino acid sequence of VH in which three modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Lys at position 12 with Val, Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Val at position 20 with Leu, Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Ala at position 40 with Arg and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys, Met at position 48 with Ile and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;

[0080] Specific examples of the amino acid sequence of VH in which two modifications are introduced include the following amino acid sequence (1) to (21):

(1) an amino acid sequence in which substitutions of Lys at position 12 with Val and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitutions of Val at position 20 with Leu and Arg at position 38 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(3) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ala at position 40 with Arg are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitutions of Arg at position 38 with Lys and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(12) an amino acid sequence in which substitutions of Lys at position 12 with Val and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(13) an amino acid sequence in which substitutions of Val at position 20 with Leu and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(14) an amino acid sequence in which substitutions of Ala at position 40 with Arg and Met at position 48 with Ile are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(15) an amino acid sequence in which substitutions of Met at position 48 with Ile and Arg at position 67 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(16) an amino acid sequence in which substitutions of Met at position 48 with Ile and Val at position 68 with Ala are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(17) an amino acid sequence in which substitutions of Met at position 48 with Ile and Ile at position 70 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(18) an amino acid sequence in which substitutions of Met at position 48 with Ile and Ala at position 72 with Val are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(19) an amino acid sequence in which substitutions of Met at position 48 with Ile and Thr at position 74 with Lys are introduced in the amino acid sequence represented by SEQ ID NO: 67;
(20) an amino acid sequence in which substitutions of Met at position 48 with Ile and Arg at position 98 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(21) an amino acid sequence in which substitutions of Met at position 48 with Ile and Val at position 113 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0081] Specific examples of the amino acid sequence of VH in which one modification is introduced include the following amino acid sequence (1) to (12):

(1) an amino acid sequence in which substitution of Lys at position 12 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(2) an amino acid sequence in which substitution of Val at position 20 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67;

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(3) an amino acid sequence in which substitution of Arg at position 38 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(4) an amino acid sequence in which substitution of Ala at position 40 with Arg is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(5) an amino acid sequence in which substitution of Met at position 48 with Ile is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(6) an amino acid sequence in which substitution of Arg at position 67 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(7) an amino acid sequence in which substitution of Val at position 68 with Ala is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(8) an amino acid sequence in which substitution of Ile at position 70 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(9) an amino acid sequence in which substitution of Ala at position 72 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(10) an amino acid sequence in which substitution of Thr at position 74 with Lys is introduced in the amino acid sequence represented by SEQ ID NO: 67;
(11) an amino acid sequence in which substitution of Arg at position 98 with Gly is introduced in the amino acid sequence represented by SEQ ID NO: 67; and
(12) an amino acid sequence in which substitution of Val at position 113 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 67.

[0082] As the VL comprised in the humanized antibody, the following (1) to (6) are preferable:

(1) VL comprising an amino acid sequence in which Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(2) VL comprising an amino acid sequence in which Leu at position 11, Val at position 15, Tyr at position 36, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(3) VL comprising an amino acid sequence in which Val at position 15, Tyr at position 36, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(4) VL comprising an amino acid sequence in which Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46 and Thr at position 85 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(5) VL comprising an amino acid sequence in which Val at position 15, Tyr at position 36, Leu at position 46 and Phe at position 71 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.
(6) VL comprising an amino acid sequence in which Tyr at position 36 and Pro at position 44 in the amino acid sequence represented by SEQ ID NO: 69 are substituted with other amino acid residues.

[0083] The amino acid sequence of the above VL obtained by the amino acid modifications includes an amino acid sequence in which at least one modification among amino acid modifications for substituting Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr, and Thr at position 85 with Asp is introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0084] Examples of the amino acid sequence of VL in which nine modifications are introduced include an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr, and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0085] Specific examples of the amino acid sequence of VL in which eight modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

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(2) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(3) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(7) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(8) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and

(9) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0086] Specific example of the amino acid sequence of VL in which seven modifications are introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(3) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(7) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;

(8) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at

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position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(9) an amino acid sequence in which substitutions of Leu at position 11 with Met, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0087] Specific example of the amino acid sequence of VL in which six modifications are introduced include the following amino acid sequence (1) to (6):

(1) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(6) an amino acid sequence in which substitutions of Leu at position 11 with Met, Ala at position 13 with Val, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0088] Specific example of the amino acid sequence of VL in which five modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0089] Specific example of the amino acid sequence of VL in which four modifications are introduced include the following amino acid sequence (1) to (10):

(1) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ

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ID NO: 69;
(2) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Leu at position 46 with Gly and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(9) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(10) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0090] Specific example of the amino acid sequence ofVL in which three modifications are introduced include the following amino acid sequence (1) to (7):

(1) an amino acid sequence in which substitutions of Leu at position 11 with Met, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Ala at position 13 with Val, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Val at position 15 with Leu, Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Ala at position 43 with Ser and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu, Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0091] Specific example of the amino acid sequence of VL in which two modifications are introduced include the following amino acid sequence (1) to (15):

(1) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitutions of Leu at position 11 with Met and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitutions of Ala at position 13 with Val and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitutions of Val at position 15 with Leu and Tyr at position 36 with Leu are introduced in the amino acid sequence represented by SEQ ID NO: 69;

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(5) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Ala at position 43 with Ser are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitutions of Tyr at position 36 with Leu and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(9) an amino acid sequence in which substitutions of Leu at position 11 with Met and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(10) an amino acid sequence in which substitutions of Ala at position 13 with Val and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(11) an amino acid sequence in which substitutions of Val at position 15 with Leu and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(12) an amino acid sequence in which substitutions of Ala at position 43 with Ser and Pro at position 44 with Phe are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(13) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Leu at position 46 with Gly are introduced in the amino acid sequence represented by SEQ ID NO: 69;
(14) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Phe at position 71 with Tyr are introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(15) an amino acid sequence in which substitutions of Pro at position 44 with Phe and Thr at position 85 with Asp are introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0092] Specific example of the amino acid sequence of VH in which one modification is introduced include the following amino acid sequence (1) to (9):

(1) an amino acid sequence in which substitution of Leu at position 11 with Met is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(2) an amino acid sequence in which substitution of Ala at position 13 with Val is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(3) an amino acid sequence in which substitution of Val at position 15 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(4) an amino acid sequence in which substitution of Tyr at position 36 with Leu is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(5) an amino acid sequence in which substitution of Ala at position 43 with Ser is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(6) an amino acid sequence in which substitution of Pro at position 44 with Phe is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(7) an amino acid sequence in which substitution of Leu at position 46 with Gly is introduced in the amino acid sequence represented by SEQ ID NO: 69;
(8) an amino acid sequence in which substitution of Phe at position 71 with Tyr is introduced in the amino acid sequence represented by SEQ ID NO: 69; and
(9) an amino acid sequence in which substitution of Thr at position 85 with Asp is introduced in the amino acid sequence represented by SEQ ID NO: 69.

[0093] In addition, examples of the humanized antibody of the present invention include a humanized antibody in which VH of the antibody comprises the amino acid sequence represented by SEQ ID NO: 67 and/or VL of the antibody comprises the amino acid sequence represented by SEQ ID: 69, a humanized antibody in which VH of the antibody comprises the amino acid sequence represented by SEQ ID NO: 67 and/or VL of the antibody comprises any one of the amino acid sequences shown in Fig. 2, or a humanized antibody in which VH of the antibody comprises any one of the amino acid sequences shown in Fig. 1 and/or VL of the antibody comprises the amino acid sequence represented by SEQ ID: 69.

[0094] Furthermore, examples of the humanized antibody of the present invention include a humanized antibody which competes with the monoclonal antibody of the present invention in the binding of an amino acid sequence of an extracellular region of human TIM-3 or a three-dimensional structure thereof; and a humanized antibody which binds to the same epitope existing on the extracellular region of human TIM-3 to which the above humanized antibody binds.

[0095] A human antibody is originally an antibody naturally existing in the human body, and it also includes an antibody obtained from a human antibody phage library or a human antibody-producing transgenic animal, which is prepared

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based on the recent advanced techniques in genetic engineering, cell engineering and developmental engineering.

[0096] The antibody existing in the human body can be prepared, for example, by isolating a human peripheral blood lymphocyte, immortalizing it by infecting with EB virus or the like, and then cloning it to thereby obtain lymphocytes capable of producing the antibody, culturing the lymphocytes thus obtained, and purifying the antibody from the supernatant of the culture.

[0097] The human antibody phage library is a library in which antibody fragments such as Fab and scFv are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the index. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

[0098] A human antibody-producing transgenic animal is an animal in which a human antibody gene is integrated into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a gene encoding a human antibody into a mouse ES cell, grafting the ES cell into an early embryo of mouse and then developing it. A human antibody is prepared from the human antibody-producing transgenic non-human animal by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in non-human mammals, culturing the obtained hybridoma and forming and accumulating the human antibody in the supernatant of the culture.

[0099] In the amino acid sequence constituting the above antibody or antibody fragment thereof, an antibody or antibody fragment thereof in which one or more amino acids are deleted, substituted, inserted or added, having activity similar to the above antibody or antibody fragment thereof is also included in the antibody or antibody fragment of the present invention.

[0100] The number of amino acids which are deleted, substituted, inserted and/or added is one or more, and is not specifically limited, but it is within the range where deletion, substitution or addition is possible by known methods such as the site-directed mutagenesis described in *Molecular Cloning,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* John Wiley & Sons (1987-1997); *Nucleic Acids Research,* 10, 6487 (1982), *Proc. Natl. Acad. Sci. USA,* 79, 6409 (1982); *Gene,* 34, 315 (1985), *Nucleic Acids Research,* 13, 4431 (1985); *Proc. Natl. Acad. Sci. USA,* 82, 488 (1985) or the like. For example, the number is preferably 1 to dozens, more preferably 1 to 20, furthermore preferably 1 to 10, and most preferably 1 to 5.

[0101] The expression "one or more amino acid residue(s) is/are deleted, substituted, inserted and/or added" in the amino acid sequence of the above antibody means the followings. That is, it means there is deletion, substitution, insertion or addition of one or plural amino acids at optional positions in the same sequence and in one or plural amino acid sequences. Also, the deletion, substitution, insertion or addition may occur at the same time and the amino acid which is substituted, inserted or added may be either a natural type or a non-natural type.

[0102] The natural type amino acid includes L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-cysteine and the like.

[0103] Preferable examples of mutually substitutable amino acids are shown below. The amino acids in the same group are mutually substitutable.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine

Group B: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid

Group C: asparagine, glutamine

Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid

Group E: proline, 3-hydroxyproline, 4-hydroxyproline

Group F: serine, threonine, homoserine

Group G: phenylalanine, tyrosine

[0104] The antibody fragment of the present invention includes Fab, $F(ab')_2$, Fab', a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv), a peptide comprising CDR and the like.

[0105] An Fab is an antibody fragment among fragments obtained by treating an IgG antibody molecule with a protease, papain (cleaved at an amino acid residue at position 224 of the H chain), having a molecular weight of about 50,000 and having antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain are bound together through a disulfide bond.

[0106] The Fab of the present invention can be produced by treating the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with papain. Also, the Fab can be produced by inserting DNA encoding Fab of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to express the Fab.

[0107] An $F(ab')_2$ is an antibody fragment having a molecular weight of about 100,000 and having antigen binding

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activity and comprising two Fab regions which are bound in the hinge position obtained by digesting the lower part of two disulfide bonds in the hinge region of IgG with an enzyme, pepsin.

[0108] The $F(ab')_2$ of the present invention can be produced by treating the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with pepsin. Also, the $F(ab')_2$ can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

[0109] An Fab' is an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, which is obtained by cleaving a disulfide bond at the hinge region of the above $F(ab')_2$. The Fab' of the present invention can be produced by treating the $F(ab')_2$ of the present invention which specifically recognizes TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof with a reducing agent, such as dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to express the Fab'.

[0110] An scFv is a VH-P-VL or VL-P-VH polypeptide in which one chain VH and one chain VL are linked using an appropriate peptide linker (hereinafter referred to as "P") and is an antibody fragment having antigen binding activity.

[0111] The scFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv.

[0112] A diabody is an antibody fragment wherein scFv is dimerized, is an antibody fragment having divalent antigen binding activity. In the divalent antigen binding activity, two antigens may be the same or different.

[0113] The diabody of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding scFv so that the length of the amino acid sequence of the peptide linker is 8 or less residues, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the diabody.

[0114] A dsFv is obtained by binding polypeptides in which one amino acid residue of each of VH and VL is substituted with a cysteine residue via a disulfide bond between the cysteine residues. The amino acid residue to be substituted with a cysteine residue can be selected based on a three-dimensional structure estimation of the antibody in accordance with a known methods *[*Protein Engineering, 7, 697 (1994)].

[0115] The dsFv of the present invention can be produced by obtaining cDNAs encoding VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, constructing DNA encoding dsFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the dsFv.

[0116] A peptide comprising CDR is constituted by including one or more regions of CDRs of VH or VL. Peptide comprising plural CDRs can be bound directly or via an appropriate peptide linker.

[0117] The peptide comprising CDR of the present invention can be produced by constructing DNA encoding CDRs of VH and VL of the monoclonal antibody of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the peptide. The peptide comprising CDR can also be produced by a chemical synthesis method such as Fmoc method or tBoc method.

[0118] The monoclonal antibody of the present invention includes an antibody conjugate in which the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof is chemically or genetically bound to a radioisotope, an agent having a low molecular weight, an agent having a high molecular weight, a protein, a therapeutic antibody or the like.

[0119] The antibody conjugate of the present invention can be produced by chemically conjugating a radioisotope, an agent having a low molecular weight, an agent having a high molecular weight, an adjuvant, a protein, a therapeutic antibody or the like to the N-terminal side or C-terminal side of an H chain or an L chain, adequate substituent or sidechain, sugarchain, and the like of the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof *[*Kotai Kogaku Nyumon, published by Chijin Shokan (1994)].

[0120] Also, the antibody conjugate can be genetically produced by linking a DNA encoding the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid

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sequence of the extracellular region or three-dimensional structure thereof to other DNA encoding a protein or a therapeutic antibody to be conjugated, inserting the DNA into a vector for expression, and introducing the expression vector into an appropriate host cell.

[0121] The radioisotope includes ^{131}I, ^{125}I, ^{90}Y, ^{64}Cu, ^{199}Tc, ^{77}Lu, ^{211}At and the like. The radioisotope can directly be conjugated with the antibody by Chloramine-T method or the like. Also, a substance chelating the radioisotope can be conjugated with the antibody. The chelating agent includes 1-isothiocyanatobenzyl-3-methyldiethylenetriaminepentaacetic acid (MX-DTPA) and the like.

[0122] The agent having a low molecular weight includes an anti-tumor agent such as an alkylating agent, a nitrosourea agent, a metabolism antagonist, an antibiotic substance, an alkaloid derived from a plant, a topoisomerase inhibitor, an agent for hormonotherapy, a hormone antagonist, an aromatase inhibitor, a P glycoprotein inhibitor, a platinum complex derivative, an M-phase inhibitor and a kinase inhibitor *[*Rinsho Syuyo-gaku (Clinical Oncology), Gan to Kagakuryoho-Sha (1996)], a steroid agent such as hydrocortisone and prednisone, a nonsteroidal agent such as aspirin and indomethacin, immunomodulatory agent such as aurothiomalate, penicillamine, immuno-suppressing agent such as cyclophosphamide and azathioprine, antiinflammatory agent such as anti-histamine agent, for example, chlorpheniramine maleate and clemastine *[*Ensho to Kouensho-Ryoho (Inflammation and Anti-inflammation Therapy), Ishiyaku Shuppann (1982)] and the like.

[0123] Examples of the antitumor agent include amifostine (Ethyol), cisplatin, dacarbazine (DTIC), dactinomycin, mecloretamin (nitrogen mustard), streptozocin, cyclophosphamide, iphosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), epirubicin, gemcitabine (Gemsal), daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, fluorouracil, vinblastine, vincristine, bleomycin, daunomycin, peplomycin, estramustine, paclitaxel (Taxol), docetaxel (Taxotea), aldesleukin, asparaginase, busulfan, carboplatin, oxaliplatin, nedaplatin, cladribine, camptothecin, 10-hydroxy-7-ethylcamptothecin (SN38), floxuridine, fludarabine, hydroxyurea, iphosphamide, idarubicin, mesna, irinotecan (CPT-11), nogitecan, mitoxantrone, topotecan, leuprolide, megestrol, melfalan, mercaptopurine, hydroxycarbamide, plicamycin, mitotane, pegasparagase, pentostatin, pipobroman, streptozocin, tamoxifen, goserelin, leuprorelin, flutamide, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil, hydrocortisone, prednisolone, methylprednisolone, vindesine, nimustine, semustine, capecitabine, Tomudex, azacytidine, UFT, oxaliplatin, gefitinib (Iressa), imatinib (STI 571), elrotinib, FMS-like tyrosine kinase 3 (Flt3) inhibitor, vascular endothelial growth facotr receptor (VEGFR) inhibitor, fibroblast growth factor receptor (FGFR) inhibitor, Epidermal Growth Factor Receptor (EGFR) inhibitor such as Iressa and Tarceva, radicicol, 17-allylamino-17-demethoxygeldanamycin, rapamycin, amsacrine, all-trans-retinoic acid, thalidomide, anastrozole, fadrozole, letrozole, exemestane, gold thiomalate, D-penicillamine, bucillamine, azathioprine, mizoribine, cyclosporine, rapamycin, hydrocortisone, bexarotene (Targretin), tamoxifen, dexamethasone, progestin substances, estrogen substances, anastrozole (Arimidex), Leuplin, aspirin, indomethacin, celecoxib, azathioprine, penicillamine, gold thiomalate, chlorpheniramine maleate, chlorpheniramine, clemastine, tretinoin, bexarotene, arsenic, voltezomib, allopurinol, calicheamicin, ibritumomab tiuxetan, Targretin, ozogamine, clarithromycin, leucovorin, ifosfamide, ketoconazole, aminoglutethimide, suramin, methotrexate, maytansinoid and derivatives thereof.

[0124] The method for conjugating the agent having low molecular weight with the antibody includes a method in which the agent and an amino group of the antibody are conjugated through glutaraldehyde, a method in which an amino group of the agent and a carboxyl group of the antibody are conjugated through water-soluble carbodiimide, and the like.

[0125] The agent having a high molecular weight includes polyethylene glycol (hereinafter referred to as "PEG"), albumin, dextran, polyoxyethylene, styrene-maleic acid copolymer, polyvinylpyrrolidone, pyran copolymer, hydroxypropylmethacrylamide, and the like. By binding these compounds having a high molecular weight to an antibody or antibody fragment, the following effects are expected: (1) improvement of stability against various chemical, physical or biological factors, (2) remarkable prolongation of half life in blood, (3) disappearance of immunogenicity or suppression of antibody production, and the like *[*Bioconjugate Drug, Hirokawa Shoten (1993)]. For example, the method for binding PEG to an antibody includes a method in which an antibody is allowed to react with a PEG-modifying reagent [Bioconjugate Drug, Hirokawa Shoten (1993)]. The PEG-modifying reagent includes a modifying agent of ε-amino group of lysine (Japanese Published Unexamined Patent Application No. 178926/86), a modifying agent of a carboxyl group of aspartic acid and glutamic acid (Japanese Published Unexamined Patent Application No. 23587/81), a modifying agent of a guanidino group of arginine (Japanese Published Unexamined Patent Application No. 117920/90) and the like.

[0126] The immunostimulator may be any natural products known as immunoadjuvants. Examples of an agent enhancing immunogen include $\beta(1\rightarrow3)$glucan (lentinan, schizophyllan), α-galactosylceramide and the like.

[0127] The protein includes a cytokine or a growth factor which activates a immunocompetent cell, such as NK cell, macrophage or neutrophil, a toxic protein, and the like.

[0128] Examples of the cytokine or the growth factor include interferon (hereinafter referred to as "IFN")-α, IFN-β, IFN-γ, interleukin (hereinafter referred to as "IL")-2, IL-12, IL-15, IL-18, IL-21, IL-23, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) and the like.

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[0129] The toxic protein includes ricin, diphtheria toxin, ONTAK and the like, and also includes a toxic protein wherein mutation is introduced into a protein in order to control the toxicity.

[0130] The therapeutic antibody includes an antibody against an antigen in which apoptosis is induced by binding of the antibody, an antibody against an antigen participating in formation of pathologic state of tumor, an antibody against an antigen regulating immunological function and an antibody against an antigen relating to angiogenesis in the pathologic part.

[0131] The antigen in which apoptosis is induced by binding of the antibody includes cluster of differentiation (hereinafter "CD") 19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85, CD86 (B7.2), human leukocyte antigen (HLA)-Class II, epidermal growth factor receptor (EGFR) and the like.

[0132] The antigen participating in formation of pathologic state of tumor or the antigen regulating immunological function includes CD40, CD40 ligand, B7 family molecule (CD80, CD86, CD274, B7-DC, B7-H2, B7-H3, B7-H4), ligand of B7 family molecule (CD28, CTLA-4, ICOS, PD-1, BTLA), OX-40, OX-40 ligand, CD137, tumor necrosis factor (TNF) receptor family molecule (DR4, DR5, TNFR1, TNFR2), TNF-related apoptosis-inducing ligand receptor (TRAIL) family molecule, receptor family of TRAIL family molecule (TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4), receptor activator of nuclear factor kappa B (RANK), RANK ligand, CD25, folic acid receptor 4, cytokine [IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, transforming growth factor (TGF) β, TNFα, etc.], receptors of these cytokines, chemokine (SLC, ELC, I-309, TARC, MDC, CTACK, *etc.*) and receptors of these chemokines.

[0133] The antigen for the antibody which inhibits angiogenesis in the pathologic part includes vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), EGF, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), erythropoietin (EPO), TGFβ, IL-8, Ephilin, SDF-1, receptors thereof and the like.

[0134] A fusion antibody with a protein or therapeutic antibody can be produced by linking a cDNA encoding a monoclonal antibody or antibody fragment to a cDNA encoding the protein, constructing a DNA encoding the fusion antibody, inserting the DNA into an expression vector for prokaryote or eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the fusion antibody.

[0135] In the case where the above antibody conjugate is used for the detection method, method for quantitative determination, detection reagent, reagent for quantitative determination or diagnostic agent in the present invention, examples of the agent to which the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof is bound includes a label used in routine immunological detecting or measuring method.

[0136] The label includes enzymes such as alkaline phosphatase, peroxidase and luciferase, luminescent materials such as acridinium ester and lophine, fluorescent materials such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (RITC), and the like.

[0137] Furthermore, the present invention includes an agent for treating a disease relating to a TIM-3 positive cell, comprising the monoclonal antibody of the present invention or the antibody fragment thereof as an active ingredient.

[0138] The disease relating to the TIM-3 positive cell is not limited, so long as it is a disease relating to a cell expressing TIM-3, such as cancer, autoimmune disease, and allergic disease.

[0139] The cancer includes blood cancer, breast cancer, uterine cancer, colorectal cancer, esophageal cancer, gastric cancer, ovarian cancer, lung cancer, renal cancer, rectal cancer, thyroid cancer, uterine cervix cancer, small intestinal cancer, prostate cancer and pancreatic cancer. Preferable examples of the cancer include blood cancer, esophageal cancer, gastric cancer, colorectal cancer, liver cancer and prostate cancer.

[0140] Examples of the blood cancer include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplasticsyndromes (MDS), multiple myeloma, cutaneous T cell lymphoma (CTCL), peripheral T-cell lymphoma (PTCL), anaplastic large cell lymphoma (ALCL), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), other lymphoid leukemia, NK cell lymphoma, Hodgkin lymphoma, non-Hodgkin's lymphoma such as Burkitt's lymphoma, and the like.

[0141] Specific examples of the autoimmune diseases include rheumatoid arthritis, psoriasis, Crohn's disease, Ankylosing spondylitis, multiple sclerosis, type I diabetes, hepatitis, myocarditis, Sjogren's syndrome, autoimmune hemolytic anemia after transplant rejection, blisters pemphigoid, Graves disease, Hashimoto's thyroiditis, systemic lupus erythematosus (SLE), myasthenia gravis, pemphigus, pernicious anemia, and the like.

[0142] Examples of the allergic diseases include acute or chronic reactive airway disease, bronchial asthma, atopic dermatitis, allergic rhinitis, urticaria, PIE syndrome, food allergies, hay fever, allergic nose, bronchial asthma, atopic dermatitis, anaphylactic shock and the like.

[0143] The therapeutic agent of the present invention comprises the above monoclonal antibody or the antibody fragment of the present invention as an active ingredient.

[0144] The therapeutic agent comprising the monoclonal antibody of the present invention or antibody fragment thereof, or conjugate thereof may comprise only the antibody or antibody fragment thereof, or conjugate thereof as an active ingredient. It is generally preferred that the therapeutic agent is prepared as a pharmaceutical preparation produced by

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an appropriate method well known in the technical field of pharmaceutics, and by mixing it with one or more pharmaceutically acceptable carriers.

[0145] It is preferred to administer the therapeutic agent by the route that is most effective for the treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration and intravenous administration is preferred.

[0146] The pharmaceutical preparation includes sprays, capsules, tablets, powders, granules, syrups, emulsions, suppositorys, injections, ointments, tapes and the like.

[0147] Although the dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 μg/kg to 10 mg/kg per day and per adult.

[0148] Further, the present invention relates to a method for immunologically detecting or measuring TIM-3, a reagent for immunologically detecting or measuring TIM-3, a method for immunologically detecting or measuring a cell expressing TIM-3, and a diagnostic agent for diagnosing a disease relating to a TIM-3 positive cell, comprising the monoclonal antibody or the antibody fragment of the present invention which specifically recognizes human TIM-3 and binds to an amino acid sequence of the extracellular region or three-dimensional structure thereof as an active ingredient.

[0149] In the present invention, the method for detecting or measuring the amount of TIM-3 may be any known method. For example, it includes an immunological detecting or measuring method.

[0150] The immunological detecting or measuring method is a method in which an antibody amount or an antigen amount is detected or determined using a labeled antigen or antibody. Examples of the immunological detecting or measuring method include radioactive substance-labeled immunoantibody method (RIA), enzyme immunoassay (EIA or ELISA), fluorescent immunoassay (FIA), luminescent immunoassay, Western blotting method, physicochemical method and the like.

[0151] The above disease relating to a TIM-3 positive cell can be diagnosed by detecting or measuring a cell expressing TIM-3 by using the monoclonal antibody or antibody fragment of the present invention.

[0152] For the detection of the cell expressing the polypeptide, known immunological detection methods can be used, and an immunoprecipitation method, a fluorescent cell staining method, an immune tissue staining method and the like are preferably used. Also, a fluorescent antibody staining method using FMAT 8100 HTS system (Applied Biosystem) and the like can be used.

[0153] In the present invention, the living body sample to be used for detecting or measuring TIM-3 is not particularly limited, so long as it has a possibility of containing a cell expressing TIM-3, such as tissue cells, blood, blood plasma, serum, pancreatic fluid, urine, fecal matter, tissue fluid or culture fluid.

[0154] The diagnostic agent containing the monoclonal antibody of the present invention or antibody fragment thereof, or conjugate thereof may further contain a reagent for carrying out an antigen-antibody reaction or a reagent for detection of the reaction depending on the desired diagnostic method. The reagent for carrying out the antigen-antibody reaction includes a buffer, a salt, and the like. The reagent for detection includes a reagent generally used for the immunological detecting or measuring method, such as labeled secondary antibody which recognizes the monoclonal antibody, antibody fragment thereof or conjugates thereof and substrate corresponding to the labeling and the like.

[0155] A process for producing the antibody of the present invention, a method for treating the disease and a method for diagnosing the disease are specifically described below.

1. Preparation method of monoclonal antibody

(1) Preparation of antigen

[0156] TIM-3 or a cell expressing TIM-3 as an antigen can be obtained by introducing an expression vector comprising cDNA encoding a full length of TIM-3 or a partial length thereof is introduced into *Escherichia coli,* yeast, an insect cell, an animal cell or the like. In addition, TIM-3 can be purified and obatained from various human tumor cell lines, human tissue and the like which express a large amount of TIM-3. The tumor cell line and the tissue can be directly allowed to use as antigens. Furthermore, a synthetic peptide having a partial sequence of the TIM-3 can be prepared by a chemical synthesis method such as Fmoc method or tBoc method and used as an antigen.

[0157] TIM-3 used in the present invention can be produced, for example, by expressing a DNA encoding TIM-3 in a host cell using a method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) or the like according to the following method.

[0158] Firstly, a recombinant vector is prepared by inserting a full length cDNA comprising the region encoding TIM-3 into downstream of a promoter of an appropriate expression vector. At this time, if necessary, a DNA fragment having an appropriate length containing a region encoding the polypeptide prepared based on the full length cDNA may be used instead of the above full length cDNA. Next, a transformant producing polypeptide can be obtained by introducing

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the recombinant vector into a host cell suitable for the expression vector.

[0159] The expression vector may be any one, so long as it can replicate autonomously in the host cell to be used or it can be integrated into a chromosome comprising an appropriate promoter at such a position that the DNA encoding the polypeptide can be transcribed.

[0160] The host cell may be any one, so long as it can express the objective gene. Examples include a microorganism which belongs to the genera *Escherichia,* such as *Escherichia coli,* yeast, an insect cell, an animal cell and the like.

[0161] When a prokaryote such as *Escherichia coli* is used as the host cell, it is preferred that the recombinant vector used in the present invention is autonomously replicable in the prokaryote and comprising a promoter, a ribosome binding sequence, the DNA encoding TIM-3 and a transcription termination sequence. The recombinant vector is not necessary to have a transcription termination sequence, but a transcription termination sequence is preferably set just below the structural gene. The recombinant vector may further comprise a gene regulating the promoter.

[0162] Also, the above recombinant vector is preferably a plasmid in which the space between Shine-Dalgarno sequence, which is the ribosome binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides).

[0163] Furthermore, the nucleotide sequence of the DNA encoding TIM-3 can be substituted with another base so as to be a suitable codon for expressing in a host cell, thereby improve the productivity of the objective TIM-3.

[0164] Any expression vector can be used, so long as it can function in the host cell to be used. Examples of the expression vector includes pBTrp2, pBTac1, pBTac2 (all manufactured by Roche Diagnostics), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad Sci. USA, 82, 4306 (1985)], pBluescript II SK(-) (manufactured by Stratagene), pTrs30 [prepared from *Escherichia coli* JM109/pTrS30 (FERM BP-5407)], pTrs32 [prepared from *Escherichia coli* JM109/pTrS32 (FERM BP-5408)], pGHA2 [prepared from *Escherichia coli* IGHA2 (FERM BP-400), Japanese Published Unexamined Patent Application No. 221091/85], pGKA2 [prepared from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/85], pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (manufactured by Pharmacia), pET system (manufactured by Novagen), pME18SFL3 and the like.

[0165] Any promoter can be used, so long as it can function in the host cell to be used. Examples include promoters derived from *Escherichia coli,* phage and the like, such as trp promoter (Ptrp), lac promoter, PL promoter, PR promoter and T7 promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two Ptrp are tandemly linked, tac promoter, lacT7 promoter and letI promoter, can be used.

[0166] Examples of host cell include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* DH5α and the like.

[0167] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA into the host cell, and examples include a method using a calcium ion described in Proc. Natl. Acad. Sci. USA, 69, 2110 (1972), Gene, 17, 107 (1982) and Molecular & General Genetics, 168, 111 (1979) and the like.

[0168] When an animal cell is used as the host cell, any expression vector can be used, so long as it can function in the animal cell. Examples include pcDNAI, pcDM8 (manufactured by Funakoshi), pAGE107 [Japanese Published Unexamined Patent Application No. 22979/91; Cytotechnology, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [Nature, 329, 840,(1987)], pcDNAI/Amp (manufactured by Invitrogen), pcDNA3.1 (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J. Biochemistry, 101, 1307 (1987)], pAGE210, pME18SFL3, pKANTEX93 (WO 97/10354) and the like.

[0169] Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of immediate early (IE) gene of cytomegalovirus (CMV), SV40 early promoter, a promoter of retrovirus, a metallothionein promoter, a heat shock promoter, SRα promoter, Molony murine leukemia virus promoter or enhancer, and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

[0170] The host cell includes human Namalwa leukemia cell, monkey COS cell, Chinese hamster ovary (CHO) cell [Journal of Experimental Medicine, 108, 945 (1958); Proc.Natl.Acad.Sci. USA, 60, 1275 (1968);Genetics, 55, 513(1968); Chromosoma, 41, 129 (1973); Methods in Cell Science, 18, 115 (1996); Radiation Research, 148, 260 (1997); Proc.Natl.Acad.Sci. USA, 77, 4216 (1980); Proc.Natl.Acad.Sci. USA, 60, 1275 (1968); Cell, 6, 121 (1975); Molecular Cell genetics, Appendix I, II (pp.883-900)], CHO/DG44, CHO-K1 (ATCC accession NO: CCL-61), DUkXB11 (ATCC accession NO:CCL-9096), Pro-5 (ATCC accession NO: CCL-1781), CHO-S (Life Technologies, Cat#11619), Pro-3, rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also called as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14, Syrian hamster cell BHK or HBT5637 (Japanese Published Unexamined Patent Application No. 299/88) and the like.

[0171] Any introduction method of the recombinant vector can be used, so long as it is a method for introducing DNA

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into an animal cell, and examples include electroporation [Cytotechnology, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the like.

[0172] TIM-3 can be produced by culturing the transformant derived from a microorganism, an animal cell or the like having a recombinant vector comprising the DNA encoding TIM-3 in a medium to form and accumulate TIM-3 in the culture, and recovering it from the culture. The method for culturing the transformant in the medium is carried out according to the usual method used in culturing of hosts.

[0173] When TIM-3 is expressed in a cell derived from eukaryote, TIM-3 to which sugars or sugar chains bind can be obtained.

[0174] When a microorganism transformed with a recombinant vector containing an inducible promoter is cultured, an inducer can be added to the medium, if necessary. For example, isopropyl-β-D-thiogalactopyranoside or the like can be added to the medium when a microorganism transformed with a recombinant vector using *lac* promoter is cultured; or indoleacrylic acid or the like can be added thereto when a microorganism transformed with a recombinant vector using trp promoter is cultured.

[0175] When a transformant obtained using an animal cell as the host cell is cultured, the medium includes generally used RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], Dulbecco's modified MEM medium [Virology, 8, 396 (1959)] and 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)], Iscove's modified Dulbecco's medium (IMDM), the media to which fetal calf serum, *etc.* is added, and the like. Culture is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO_2. If necessary, an antibiotic such as kanamycin or penicillin can be added to the medium during the culturing.

[0176] Regarding the expression method of the gene encoding TIM-3, in addition to direct expression, secretory production, fusion protein expression and the like can be carried out according to the method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

[0177] The process for producing TIM-3 includes a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, a method of producing on a host cell outer membrane, and the like. The appropriate method can be selected by changing the host cell used and the structure of the TIM-3 produced.

[0178] When the TIM-3 is produced in a host cell or on a host cell membrane outer envelope, TIM-3 can be positively secreted extracellularly in accordance with the method of Paulson et al. [J. Biol. Chem., 264, 17619 (1989)], the method of Lowe et al. [Proc. Natl. Acad. Sci. USA, 86, 8227 (1989), Genes Develop., 4, 1288 (1990)], the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and WO 94/23021, and the like.

[0179] Also, the production amount of TIM-3 can be increased in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 utilizing a gene amplification system using a dihydrofolate reductase gene.

[0180] The resulting TIM-3 can be isolated and purified, for example, as follows.

[0181] When TIM-3 is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonicator, French press, Manton Gaulin homogenizer, dynomill or the like to obtain a cell-free extract.

[0182] The cell-free extract is centrifuged to obtain a supernatant, and a purified preparation can be obtained by subjecting the supernatant to a general protein isolation and purification techniques such as solvent extraction; salting out with ammonium sulfate etc.; desalting; precipitation with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical); cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia); hydrophobic chromatography using a resin such as butyl-Sepharose or phenyl-Sepharose; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing; and the like which may be used alone or in combination.

[0183] When TIM-3 is expressed intracellularly by forming an inclusion body, the cells are recovered, disrupted and centrifuged in the same manner, and the inclusion body of TIM-3 are recovered as a precipitation fraction. The recovered inclusion body of the TIM-3 protein is solubilized with a protein denaturing agent. The protein is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified preparation of polypeptide is obtained by the same isolation purification method as above.

[0184] When TIM-3 or the derivative such as a glycosylated product is secreted extracellularly, TIM-3 or the derivative such as a glycosylated product can be recovered from the culture supernatant. That is, the culture is treated by a method such as centrifugation in the same manner as above to obtain a soluble fraction, a purified preparation of TIM-3 can be obtained from the soluble fraction by the same isolation purification method as above.

[0185] Also, TIM-3 used in the present invention can be produced by a chemical synthesis method, such as Fmoc method or tBoc method. Also, it can be chemically synthesized using a peptide synthesizer manufactured by Advanced ChemTech, Perkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corpo-

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ration, or the like.

(2) Immunization of animal and preparation of antibody-producing cell for fusion

[0186] A mouse, rat or hamster and the like which is 3 to 20-weeks-old is immunized with the antigen prepared in the above (1), and antibody-producing cells within the spleen, lymph node or peripheral blood of the animal are collected. Also, when the increase of a sufficient titer in the above animal is not recognized due to low immunogenecity, a TIM-3 knockout mouse may by used as an animal to be immunized.

[0187] The immunization is carried out by administering the antigen to the animal through subcutaneous, intravenous or intraperitoneal injection together with an appropriate adjuvant (for example, complete Freund's adjuvant, combination of aluminum hydroxide gel with pertussis vaccine, or the like). When the antigen is a partial peptide, a conjugate is produced with a carrier protein such as BSA (bovine serum albumin), KLH (keyhole limpet hemocyanin) or the like, which is used as the antigen.

[0188] The administration of the antigen is carried out 5 to 10 times every one week or every two weeks after the first administration. On the 3rd to 7th day after each administration, a blood sample is collected from the fundus of the eye, the reactivity of the serum with the antigen is tested, for example, by enzyme immunoassay [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like. An animal showing a sufficient antibody titer in their sera against the antigen used for the immunization is used as the source of antibody-producing cells for fusion.

[0189] Three to seven days after final administration of the antigen, tissue containing the antibody-producing cells such as the spleen is excised from the immunized animal to collect the antibody-producing cells. When the spleen cells are used, the spleen is cut out and loosened, followed by centrifugation. Then, antibody-producing cells for fusion are obtained by removing erythrocytes.

(3) Preparation of myeloma cell

[0190] An established cell line obtained from mouse is used as myeloma cells. Examples include 8-azaguanine-resistant mouse (derived from BALB/c) myeloma cell line P3-X63Ag8-U1 (P3-U1) [Current Topics in Microbiology and Immunology, 18, 1 (1978)], P3-NS1/1-Ag41 (NS-1) [European J. Immunology, 6, 511 (1976)], SP2/0-Ag14 (SP-2) [Nature, 276, 269 (1978)], P3-X63-Ag8653 (653) [J. Immunology, 123, 1548 (1979)], P3-X63-Ag8 (X63) [Nature, 256, 495 (1975)] and the like.

[0191] The myeloma cells are subcultured in a normal medium [a medium in which glutamine, 2-mercaptoethanol, gentamicin, FBS and 8-azaguanine are added to RPMI-1640 medium] and they are subcultured in the normal medium 3 or 4 days before cell fusion to ensure the cell number of 2×10^7 or more on the day for fusion.

(4) Cell fusion and preparation of hybridoma for producing monoclonal antibody

[0192] The antibody-producing cells for fusion obtained by the above (2) and myeloma cells obtained by the above (3) were sufficiently washed with a minimum essential medium (MEM) or PBS (1.83 g of disodium hydrogen phosphate, 0.21 g of potassium dihydrogen phosphate, 7.65 g of sodium chloride, 1 liter of distilled water, pH 7.2) and mixed to give a ratio of the antibody-producing cells: the myeloma cells = 5 to 10: 1, followed by centrifugation. Then, the supernatant is discarded.

[0193] The precipitated cell group is sufficiently loosened. After loosening the precipitated cell, the mixture of polyethylene glycol-1000 (PEG-1000), MEM and dimethylsulfoxide is added to the cell under stirring at 37°C. In addition, 1 to 2 mL of MEM medium is added several times every one or two minutes, and MEM is added to give a total amount of 50 mL. After centrifugation, the supernatant is discarded. After the cells are gently loosened, the cells are gently suspended in HAT medium [a normal medium containing hypoxanthine, thymidine and aminopterin]. The suspension is cultured in a 5% CO_2 incubator for 7 to 14 days at 37°C.

[0194] After the culturing, a portion of the culture supernatant is sampled and a hybridoma which is reactive to an antigen containing TIM-3 and is not reactive to an antigen not containing TIM-3 is selected by binding assay as described below. Then, cloning is carried out twice by a limiting dilution method [Firstly, HT medium (HAT medium from which aminopterin is removed) is used, and secondly, the normal medium is used], and a hybridoma which stably shows a high antibody titer is selected as the monoclonal antibody-producing hybridoma.

(5) Preparation of purified monoclonal antibody

[0195] The hybridoma cells producing a monoclonal antibody obtained by the above (4) are administered by intraperitoneal injection into 8- to 10-week-old mice or nude mice treated with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane (pristane) is intraperitoneally administered, followed by feeding for 2 weeks). The hybridoma develops ascites

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tumor in 10 to 21 days. The ascitic fluid is collected from the mice, centrifuged to remove solids, subjected to salting out with 40 to 50% ammonium sulfate and then precipitated by caprylic acid, passed through a DEAE-Sepharose column, a protein A column or a gel filtration column to collect an IgG or IgM fraction as a purified monoclonal antibody.

[0196] Furthermore, a monoclonal antibody-producing hybridoma obtained by the above (4) is cultured in RPMI1640 medium containing 10% FBS or the like and the supernatant is removed by centrifugation. The precipitated cells are suspended in Hybridoma-SFM medium and cultured for 3 to 7 days. The purified monoclonal antibody can be obtained by centrifusing the obtained cell suspension, followed by purification from the resulting supernatant with Protein A column or Protein G column to collect the IgG fractions. Additionally, Hybridoma-SFM medium can contain 5% DIGO GF21.

[0197] The subclass of the antibody can be determined using a subclass typing kit by enzyme immunoassay. The amount of the protein can be determined by the Lowry method or from the absorbance at 280 nm.

(6) Selection of monoclonal antibody

[0198] Selection of monoclonal antibody is carried out by the following binding assay using an enzyme immunoassay method and inhibition assay of intracellular uptake of amino acids.

(6-a) Binding assay

[0199] As the antigen, a gene-introduced cell obtained by introducing an expression vector containing a cDNA encoding TIM-3 obtained in (1) into *Escherichia coli,* yeast, an insect cell, an animal cell or the like, a recombinant protein, or a purified polypeptide or partial peptide obtained from a human tissue is used. When the antigen is a partial peptide, a conjugate is prepared with a carrier protein such as BSA or KLH is prepared and is used.

[0200] After making these antigens into a solid layer by dispensing in a 96-well plate, a substance to be tested such as serum, a culture supernatant of a hybridoma or a purified monoclonal antibody is dispensed therein as the primary antibody and allowed to react. After thoroughly washing with PBS, PBS-Tween and the like, an anti-immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent material, a radiation compound or the like is dispensed therein as the secondary antibody and allowed to react. After thoroughly washing with PBS-Tween, the reaction appropriate to the label of the secondary antibody is carried out to select a monoclonal antibody which specifically reacts with the antigen.

[0201] In addition, a monoclonal antibody which competes with the anti-TIM-3 monoclonal antibody of the present invention in the binding of an extracellular region of human TIM-3 can be obtained by adding a subject antibody to the above binding assay system to allow to react. Namely, a monoclonal antibody which competes with the obtained monoclonal antibody in the binding of an amino acid sequence of the extracellular region of human TIM-3 or a three-dimensional structure thereof can be obtained by screening a subject antibody which inhibits the binding of the monoclonal antibody upon adding to the antibody.

[0202] Furthermore, an antibody which binds to the same epitope to which the monoclonal antibody of the present invention which binds to an amino acid sequence of the extracellular region of TIM-3 or three-dimensional structure thereof can be obtained by identifying an epitope of the antibody obtained by the above binding assay system, preparing a partial synthetic peptide of the epitope or a synthetic peptide which mimics the three-dimensional structure of the epitope and immunizing with the peptide.

(6-b) Kinetic analysis with Biacore

[0203] The kinetics between an antigen and a test substance is measured using Biacore T100 and then the obtained results are analyzed using analysis software accompanied with the apparatus. After anti-mouse IgG antibody is immobilized onto to a CM 5 sensor chip by an amine coupling method, a test substance such as culture supernatant of a hybridoma, a purified monoclonal antibody is allowed to flow and bind at an appropriate amount, and an antigen at plural known concentrations is further allowed to flow. And then, the binding and dissociation are measured.

[0204] Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using the 1:1 binding model to obtain necessary parameters. Otherwise, after human TIM-3 is immobilized onto the sensor chip by an amino coupling method, a purified monoclonal antibody is allowed to flow at plural known concentrations followed by measuring the binding and dissociation. Using the obtained data and the software accompanied with the apparatus, the kinetics analysis is carried out using bivalent analyte model to obtain necessary parameters.

2. Preparation of recombinant antibody

[0205] As production examples of recombinant antibodies, processes for producing a human chimeric antibody and a human CDR grafted antibody are shown below.

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(1) Construction of vector for expression of recombinant antibody

[0206] A vector for expression of recombinant antibody is an expression vector for animal cell into which DNAs encoding CH and CL of a human antibody have been inserted, and is constructed by cloning each of DNAs encoding CH and CL of a human antibody into an expression vector for animal cell.

[0207] The C region of a human antibody may be used CH and CL of any human antibody. Examples include CH belonging to γ1 subclass, CL belonging to κ class, and the like. As the DNAs encoding CH and CL of a human antibody, the cDNA may be generally used and a chromosomal DNA comprising an exon and an intron can be also used.

[0208] As the expression vector for animal cell, any expression vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [Cytotechnol., 3, 133 (1990)], pAGE103 [J. Biochem., 101, 1307 (1987)], pHSG274 [Gene, 27, 223 (1984)], pKCR [Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)], pSG1bd2-4 [Cytotechnol., 4, 173 (1990)], pSE1UK1Sed1-3 [Cytotechnol., 13, 79 (1993)] and the like.

[0209] Examples of a promoter and enhancer used for an expression vector for animal cell include an SV40 early promoter [J. Biochem., 101, 1307 (1987)], a Moloney mouse leukemia virus LTR [Biochem. Biophys. Res. Commun., 149, 960 (1987)], an immunoglobulin H chain promoter [Cell, 41, 479 (1985)] and enhancer [Cell, 33, 717 (1983)] and the like.

[0210] The vector for expression of recombinant antibody may be either of a type in which a gene encoding an antibody H chain and a gene encoding an antibody L chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a vector for expression of recombinant antibody, easiness of introduction into animal cells, and balance between the expression amounts of antibody H and L chains in animal cells, a tandem type of the vector for expression of recombinant antibody is more preferred [J. Immunol. Methods, 167, 271 (1994)]. Examples of the tandem type of the vector for expression of recombinant antibody include pKANTEX93 (WO 97/10354), pEE18 [Hybridoma, 17, 559 (1998)], and the like.

(2) Obtaining of cDNA encoding V region of antibody derived from non-human animal and analysis of amino acid sequence

[0211] Obtaining of cDNAs encoding VH and VL of a non-human antibody and analysis of amino acid sequence are carried out as follows.

[0212] mRNA is extracted from hybridoma cells producing a non-human antibody to synthesize cDNA. The synthesized cDNA is cloned into a vector such as a phage or a plasmid, to prepare a cDNA library.

[0213] Each of a recombinant phage or recombinant plasmid containing cDNA encoding VH or VL is isolated from the library using DNA encoding a part of the C region or V region of a mouse antibody as the probe. The full length of the nucleotide sequences of VH and VL of a mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and the full length of the amino acid sequences of VH and VL are deduced from the nucleotide sequences, respectively.

[0214] Examples of the non-human animal for preparing a hybridoma cell which produces a non-human antibody include mouse, rat, hamster, rabbit or the like. Any animals can be used so long as a hybridoma cell can be produced therefrom.

[0215] Examples of the method for preparing total RNA from a hybridoma cell include a guanidine thiocyanate-cesium trifluoroacetate method [Methods in Enzymol., 154, 3 (1987)], the use of a kit such as RNA easy kit (manufactured by Qiagen) and the like.

[0216] Examples of the method for preparing mRNA from total RNA include an oligo (dT) immobilized cellulose column method [Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)], a method using a kit such as Oligo-dT30 <Super> mRNA Purification Kit (manufactured by Takara Bio) and the like. Additionary, examples of the method for preparing mRNA include method using a kit Fast Track mRNA Isolation kit (manufactured by Invitrogen) or QuickPrep mRNA Purification Kit (manufactured by Pharmacia) and the like.

[0217] Examples of the method for synthesizing cDNA and preparing a cDNA library include known methods [*Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* Supplement 1, John Wiley & Sons (1987-1997)]; a method using a kit such as Super Script Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Invitrogen), ZAP-cDNA Synthesis Kit (manufactured by Stratagene), *etc.;* and the like.

[0218] The vector into which the synthesized cDNA using mRNA extracted from a hybridoma cell as the template is inserted for preparing a cDNA library may be any vector, so long as the cDNA can be inserted. Examples include ZAP ExPress [*Strategies, 5,* 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research,* 17, 9494 (1989)], λZAPII (manufactured by Stratagene), λgt10 and λgt11 [*DNA Cloning: A Practical Approach,* I, 49 (1985)], Lambda BlueMid (manufactured by Clontech), λExCell and pT7T3-18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.,* 3, 280 (1983)], pUC18 [*Gene,* 33, 103 (1985)], and the like.

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[0219] Any *Escherichia coli* for introducing the cDNA library constructed by a phage or plasmid vector may be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF' [*Strategies,* 5, 81 (1992)], C600 [*Genetics,* 39, 440 (1954)], Y1088 and Y1090 [*Science, 222:* 778 (1983)], NM522 [*J. Mol. Biol.,* 166, 1 (1983)], K802 [*J. Mol. Biol.,* 16, 118 (1966)], JM105 [*Gene,* 38, 275 (1985)], and the like.

[0220] A colony hybridization or plaque hybridization method using an isotope- or fluorescence-labeled probe may be used for selecting cDNA clones encoding VH or VL of a non-human antibody or the like from the cDNA library [*Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989)].

[0221] Also, the cDNAs encoding VH and VL can be prepared through polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning, A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press (1989); *Current Protocols in Molecular Biology,* Supplement 1, John Wiley & Sons (1987-1997)) by preparing primers and using cDNA prepared from mRNA or a cDNA library as the template.

[0222] The nucleotide sequence of the cDNA can be determined by digesting the cDNA selected with appropriate restriction enzymes and the like, cloning the fragments into a plasmid such as pBluescript SK(-) (manufactured by Stratagene), carrying out the reaction by a usually used nucleotide sequence analyzing method. For example, a nucleotide sequence analyzing method is carried out by using an automatic nucleotide sequence analyzer such as ABI PRISM3700 (manufactured by PE Biosystems) and A.L.F. DNA sequencer (manufactured by Pharmacia) after a reaction such as the dideoxy method *[*Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)].

[0223] Whether the obtained cDNAs encode the full amino acid sequences of VH and VL of the antibody containing a secretory signal sequence can be confirmed by estimating the full length of the amino acid sequences of VH and VL from the determined nucleotide sequence and comparing them with the full length of the amino acid sequences of VH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)].

[0224] With regard to the full amino acid sequences of VH and VL of the antibody containing a secretory signal sequence, the length of the secretory signal sequence and N-terminal amino acid sequence can be estimated by comparing them with the full length of the amino acid sequences ofVH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)], and furthermore the subgroup to which they belong can be determined. In addition, the amino acid sequence of each CDR of VH and VL can be determined by comparing them with the full length of the amino acid sequences of VH and VL of known antibodies [A.L.F. DNA sequencer, US Dept. Health and Human Services (1991)].

[0225] Moreover, the novelty of the full length of the amino acid sequence of VH and VL can be examined by carrying out a homology search with sequences in any database, for example, SWISS-PROT, PIR-Protein or the like using the obtained full length of the amino acid sequences of VH and VL, for example, according to the BLAST method [J. Mol. Biol., 215, 403 (1990)] or the like.

(3) Construction of vector for expression of human chimeric antibody

[0226] cDNA encoding each of VH and VL of antibody of non-human animal is cloned in the upstream of genes encoding CH or CL of human antibody of vector for expression of recombinant antibody mentioned in the above (1) to thereby construct a vector for expression of human chimeric antibody.

[0227] In order to ligate cDNA comprising a nucleotide sequence of 3'-terminal of VH or VL of antibody of non-human animal and a nucleotide sequence of 5'-terminal of CH or CL of human antibody, each cDNA encoding VH or VL of a non-human animal antibody is prepared so as to encodes appropriate amino acids and have an appropriate recognition sequence of a restriction enzyme at a linkage portion.

[0228] The prepared cDNAs encoding VH and VL of antibody are respectively cloned so that each of them is expressed in an appropriate form in the upstream of gene encoding CH or CL of the human antibody of the vector for expression of the human CDR-grafted antibody mentioned in the above (1) to construct a vector for expression of human chimeric antibody.

[0229] In addition, cDNA encoding VH or VL of a non-human animal antibody is amplified by PCR using a synthetic DNA having a recognition sequence of an appropriate restriction enzyme at both ends and each of them is cloned to the vector for expression of recombinant antibody obtained in the above (1).

(4) Construction of cDNA encoding V region of human CDR-grafted antibody

[0230] cDNAs encoding VH or VL of a human CDR-grafted antibody can be obtained as follows.

[0231] Amino acid sequences of framework region (hereinafter referred to as "FR") in VH or VL of a human antibody to which amino acid sequences of CDRs in VH or VL of an antibody derived from a non-human animal antibody are transplanted are respectively selected. Any amino acid sequences of FR of a human antibody can be used, so long as they are derived from human.

[0232] Examples include amino acid sequences of FRs of human antibodies registered in database such as Protein

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Data Bank or the like, and amino acid sequences common to subgroups of FRs of human antibodies [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the like. In order to inhibit the decrease in the binding activity of the antibody, amino acid sequences having high homology (at least 60% or more) with the amino acid sequence of FR in VH or VL of the original antibody is selected.

[0233] Then, amino acid sequences of CDRs of the original antibody are grafted to the selected amino acid sequence of FR in VH or VL of the human antibody, respectively, to design each amino acid sequence of VH or VL of a humanized antibody. The designed amino acid sequences are converted to DNA sequences by considering the frequency of codon usage found in nucleotide sequences of genes of antibodies [Sequence of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)], and the DNA sequence encoding the amino acid sequence of VH or VL of a humanized antibody is designed.

[0234] Based on the designed nucleotide sequences, several synthetic DNAs having a length of about 100 nucleotides are synthesized, and PCR is carried out using them. In this case, it is preferred that 6 synthetic DNAs per each of the H chain and the L chain are designed in view of the reaction efficiency of PCR and the lengths of DNAs which can be synthesized.

[0235] Furthermore, the cDNA encoding VH or VL of a human CDR-grafted antibody can be easily cloned into the vector for expression of human CDR-grafted antibody constructed in (1) by introducing the recognition sequence of an appropriate restriction enzyme to the 5' terminal of the synthetic DNAs existing on the both ends.

[0236] Otherwise, it can be carried out using a synthetic DNA as one DNA encoding each of the full-length H chain and the full-length L chain based on the designed DNA sequence.

[0237] After the PCR, an amplified product is cloned into a plasmid such as pBluescript SK (-) (manufactured by Stratagene) or the like, and the nucleotide sequence is determined according to a method similar to the method described in (2) to obtain a plasmid having a DNA sequence encoding the amino acid sequence of VH or VL of a desired humanized antibody.

(5) Modification of amino acid sequence of V region of human CDR-grafted antibody

[0238] It is known that when a human CDR-grafted antibody is produced by simply grafting only CDRs in VH and VL of an antibody derived from a non-human animal into FRs of VH and VL of a human antibody, its antigen binding activity is lower than that of the original antibody derived from a non-human animal [BIO/TECHNOLOGY, 9, 266 (1991)].

[0239] In human CDR-grafted antibodies, among the amino acid sequences of FRs in VH and VL of a human antibody, an amino acid residue which directly relates to binding to an antigen, an amino acid residue which interacts with an amino acid residue in CDR, and an amino acid residue which maintains the three-dimensional structure of an antibody and indirectly relates to binding to an antigen are identified and modified to an amino acid residue which is found in the original non-humanized antibody to thereby increase the antigen binding activity which has been decreased.

[0240] In order to identify the amino acid residues relating to the antigen binding activity in FR, constructing the three-dimensional structure of an antibody and analyzing can be done by X-ray crystallography [J. Mol. Biol., 112, 535 (1977)], computer-modeling [Protein Engineering, 7, 1501 (1994)] or the like. In addition, modified human CDR-grafted antibody having sufficient binding activity against antigen can be obtained by various attempts, such as producing several modified antibodies of each antibody and examining their binding activities.

[0241] The modification of the amino acid sequence of FR in VH and VL of a human antibody can be accomplished using various synthetic DNA for modification according to PCR as described in (4). With regard to the amplified product obtained by the PCR, the nucleotide sequence is determined according to the method as described in (2) so that whether the objective modification has been carried out is confirmed.

(6) Construction of vector for expression of human CDR-grafted antibody

[0242] A vector for expression of human CDR-grafted antibody can be constructed by cloning each cDNA encoding VH or VL of a constructed recombinant antibody into upstream of each gene encoding CH or CL of the human antibody in the vector for expression of recombinant antibody as described in (1).

[0243] For example, when recognizing sequences of an appropriate restriction enzymes are introduced to the 5'-terminal of synthetic DNAs positioned at both ends among synthetic DNAs used in the construction of VH or VL of the human CDR-grafted antibody in (4) and (5), cloning can be carried out so that they are expressed in an appropriate form in the upstream of each gene encoding CH or CL of the human antibody in the vector for expression of a human CDR-grafted antibody as described in (1).

(7) Transient expression of recombinant antibody

[0244] In order to efficiently evaluate the antigen binding activity of various human CDR-grafted antibodies produced,

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the recombinant antibodies can be expressed transiently using the vector for expression of antibody as described in (3) and (6) or the modified expression vector thereof.

[0245] Any cell can be used as a host cell, so long as the host cell can express a recombinant antibody. For example, COS-7 cell (ATCC CRL1651) is used in view of its high expression amount *[*Methods in Nucleic Acids Res., CRC Press, 283 (1991)].

[0246] Examples of the method for introducing the expression vector into COS-7 cell include a DEAE-dextran method *[Methods in Nucleic Acids Res.,* CRC Press, 283 (1991)], a lipofection method *[Proc. Natl. Acad. Sci. USA,* 84, 7413 (1987)], and the like.

[0247] After introduction of the expression vector, the expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be determined by the enzyme immunoassay [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988), Monoclonal Antibody Experiment Manual, Kodansha Scientific (1987)] and the like.

(8) Obtaining transformant which stably expresses recombinant antibody and preparation of recombinant antibody

[0248] A transformant which stably expresses a recombinant antibody can be obtained by introducing the vector for expression of recombinant antibody described in (3) and (6) into an appropriate host cell.

[0249] Examples of the method for introducing the expression vector into a host cell include electroporation [Japanese Published Unexamined Patent Application No. 257891/90, Cytotechnology, 3, 133 (1990)] and the like.

[0250] As the host cell into which a vector for expression of a recombinant antbody is introduced, any cell can be used, so long as it is a host cell which can produce the recombinant antibody. Examples include CHO-K1 (ATCC CCL-61), DUkXB11 (ATCC CCL-9096), Pro-5 (ATCC CCL-1781), CHO-S (Life Technologies, Cat # 11619), rat myeloma cell YB2/3HL.P2.G11.16Ag.20 (also referred to as YB2/0), mouse myeloma cell NSO, mouse myeloma cell SP2/0-Ag14 (ATCC No. CRL1581), mouse P3X63-Ag8653 cell (ATCC No. CRL1580), CHO cell in which a dihydrofolate reductase gene (hereinafter referred to as "dhfr") is defective [Proc. Natl. Acad. Sci. U.S.A., 77, 4216 (1980)], lection resistance-acquired Lec 13 [Somatic Cell and Molecular genetics, 12, 55 (1986)], CHO cell in which α1,6-fucosyltransaferse gene is defected (WO 2005/35586, WO 02/31140), rat YB2/3HL.P2.G11.16Ag.20 cell (ATCC No. CRL1662), and the like.

[0251] In addition, host cells in which activity of a protein such as an enzyme relating to synthesis of an intracellular sugar nucleotide, GDP-fucose, a protein such as an enzyme relating to the modification of a sugar chain in which 1-position of fucose is bound to 6-position of N-acetylglucosamine in the reducing end through α-bond in a complex type N-glycoside-linked sugar chain, or a protein relating to transport of an intracellular sugar nucleotide, GDP-fucose, to the Golgi body is decreased or deleted, preferably CHO cell in which α1,6-fucosyltransferase gene is defected as described in W02005/35586, WO02/31140 or the like, can also be used.

[0252] After introduction of the expression vector, transformants which express a recombinant antibody stably are selected by culturing in a medium for animal cell culture containing an agent such as G418 sulfate (hereinafter referred to as "G418") or the like (Japanese Published Unexamined Patent Application No. 257891/90).

[0253] Examples of the medium for animal cell culture include RPMI1640 medium (manufactured by Invitrogen), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL301 medium (manufactured by JRH), IMDM medium (manufactured by Invitrogen), Hybridoma-SFM medium (manufactured by Invitrogen), media obtained by adding various additives such as FBS to these media, and the like.

[0254] The recombinant antibody can be produced and accumulated in a culture supernatant by culturing the obtained transformants in a medium. The expression amount and antigen binding activity of the recombinant antibody in the culture supernatant can be measured by ELISA or the like. Also, in the transformant, the expression amount of the recombinant antibody can be increased by using DHFR amplification system or the like according to the method disclosed in Japanese Published Unexamined Patent Application No. 257891/90.

[0255] The recombinant antibody can be purified from the culture supernatant of the transformant by using a protein A column [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)]. In addition, the recombinant antibody can be purified by a combination of the methods for purification such as gel filtration, ionexchange chromatography, ultrafiltration and the like.

[0256] The molecular weight of the H chain or the L chain of the purified recombinant antibody or the antibody molecule as a whole is determined by polyacrylamide gel electrophoresis (hereinafter referred to as "SDS-PAGE") *[*Nature, 227, 680 (1970)], Western blotting [Monoclonal Antibodies-Principles and practice, Third edition, Academic Press (1996), Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], and the like.

3. Activity evaluation of the monoclonal antibody or antibody fragment

[0257] The activity of the purified monoclonal antibody or antibody fragment of the present invention can be evaluated in the following manner.

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[0258] The binding activity to TIM-3-expressing cell is evaluated by the binding assay described in the above 1-(6a) and a surface plasmon resonance method using such as the Biacore system described in the above (6b). Furthermore, it can be measured by fluorescent antibody technique [Cancer Immunol. Immunother, 36, 373 (1993)] and the like.

[0259] Complement-Dependent Cytotoxicity (hereinafter, referred to as "CDC activity") or ADCC activity against an antigen positive cell line is evaluated by a known method [Cancer Immunol. Immunother., 36, 373 (1993)].

4. Method of controlling effector activity of antibody

[0260] As a method for controlling an effector activity of the monoclonal antibody of the present invention, a method for controlling an amount of fucose (hereinafter, referred to also as "core fucose") which is bound in α-1,6 linkage to N-acetylglucosamine (G1cNAc) present in a reducing end of a complex type N-linked sugar chain which is bound to asparagine (Asn) at position 297 of an Fc region of an antibody (W02005/035586, W02002/31140, and WO00/61739), a method for controlling an effector activity of a monoclonal antibody by modifying amino acid group(s) of an Fc region of the antibody, and the like are known. The effector activity of the anti-TIM-3 monoclonal antibody of the present invention can be controlled by using any of the methods.

[0261] The "effector activity" means an antibody-dependent activity which is induced via an Fc region of an antibody. As the effector activity, ADCC activity, CDC activity, an antibody-dependent phagocytosis (ADP activity) by phagocyte such as macrophages or dendritic cells, and the like are known.

[0262] By controlling a content of core fucose of a complex type N-linked sugar chain of Fc of an antibody, an effector activity of the antibody can be increased or decreased. According to a method for lowering a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of the antibody, an antibody to which fucose is not bound can be obtained by the expression of an antibody using a CHO cell which is deficient in a gene encoding α1,6-fucosyl-transferase. The antibody to which fucose is not bound has a high ADCC activity.

[0263] On the other hand, according to a method for increasing a content of fucose which is bound to a complex type N-linked sugar chain bound to Fc of an antibody, an antibody to which fucose is bound can be obtained by the expression of an antibody using a host cell into which a gene encoding α1,6-fucosyltransferase is introduced. The antibody to which fucose is bound has a lower ADCC activity than the antibody to which fucose is not bound.

[0264] Further, by modifying amino acid residue(s) in an Fc region of an antibody, the ADCC activity or CDC activity can be increased or decreased. For example, the binding activity to an antibody can be increased by using the amino acid sequence of the Fc region described in US2007/0148165. Further, the ADCC activity or CDC activity can be increased or decreased by modifying the amino acid as described in US Patent Nos. 6,737,056, or 7,297,775 or 7,317,091.

[0265] Furthermore, an antibody in which the effector activity is controlled can be obtained by combining the above method within one antibody.

5. Method for treating disease using the anti-TIM-3 monoclonal antibody or antibody fragment of the present invention

[0266] The monoclonal antibody or an antibody fragment thereof of the present invention can be used for treating a disease relating to a TIM-3 positive cell.

[0267] The therapeutic agent comprising the antibody or antibody fragment of the present invention or derivatives thereof may be only the antibody or antibody fragment or derivatives thereof as an active ingredient, and is preferably supplied as a pharmaceutical preparation produced by an appropriate method well known in the technical field of pharmaceutics, by mixing it with one or more pharmaceutically acceptable carriers.

[0268] Examples of a route of administration include oral administration and non-oral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular or intravenous administration. Examples of the dosage form includes sprays, capsules, tablets, powder, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

[0269] The pharmaceutical preparation suitable for oral administration includes emulsions, syrups, capsules, tablets, powders, granules and the like.

[0270] Liquid preparations such as emulsions and syrups can be produced using, as additives, water; sugars such as sucrose, sorbitol and fructose; glycols such as polyethylene glycol and propylene glycol; oils such as sesame oil, olive oil and soybean oil; antiseptics such as p-hydroxybenzoic acid esters; flavors such as strawberry flavor and peppermint; and the like.

[0271] Capsules, tablets, powders, granules and the like can be produced using, as additives, excipients such as lactose, glucose, sucrose and mannitol; disintegrating agents such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose and gelatin; surfactants such as fatty acid ester; plasticizers such as glycerin; and the like.

[0272] The pharmaceutical preparation suitable for parenteral administration includes injections, suppositories, sprays and the like.

[0273] Injections can be prepared using a carrier such as a salt solution, a glucose solution or a mixture of both thereof.

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[0274] Suppositories can be prepared using a carrier such as cacao butter, hydrogenated fat or carboxylic acid.

[0275] Sprays can be prepared using the antibody or antibody fragment as such or using it together with a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the compound by dispersing it as fine particles. The carrier includes lactose, glycerol and the like. It is possible to produce pharmaceutical preparations such as aerosols and dry powders.

[0276] In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

6. Method for diagnosing disease using the anti-TIM-3 monoclonal antibody or antibody fragment thereof in the present invention

[0277] A disease relating to TIM-3 can be diagnosed by detecting or determining TIM-3 or a cell expressing TIM-3 using the monoclonal antibody or antibody fragment of the present invention.

[0278] A diagnosis of cancer which is one of the diseases relating to TIM-3 can be carried out by, for example, the following detection or measurement of TIM-3.

[0279] The diagnosis of cancer can be carried out by detecting TIM-3 expression on the cell in a patient's body by an immunological method such as a flow cytometry.

[0280] An immunological method is a method in which an antibody amount or an antigen amount is detected or determined using a labeled antigen or antibody. Examples of the immunological method include radioactive substance-labeled immunoantibody method, enzyme immunoassay, fluorescent immunoassay, luminescent immunoassay, Western blotting method, physico-chemical means and the like.

[0281] Examples of the radioactive substance-labeled immunoantibody method include a method, in which the antibody or antibody fragment of the present invention is allowed to react with an antigen, a cell expressing an antigen or the like, then anti-immunoglobulin antibody subjected to a radioactive labeling or a binding fragment thereof is allowed to react therewith, followed by determination using a scintillation counter or the like.

[0282] Examples of the enzyme immunoassay include a method, in which the antibody or antibody fragment of the present invention is allowed to react with an antigen, a cell expressing an antigen or the like, then an anti-immunoglobulin antibody or an binding fragment thereof subjected to antibody labeling is allowed to react therewith and the colored pigment is measured by a spectrophotometer, and, for example, sandwich ELISA may be used.

[0283] As a label used in the enzyme immunoassay, any known enzyme label (Enzyme Immunoassay, published by Igaku Shoin, 1987) can be used as described previously. Examples include alkaline phosphatase labeling, peroxidase labeling, luciferase labeling, biotin labeling and the like.

[0284] Sandwich ELISA is a method in which an antibody is bound to a solid phase, antigen to be detected or measured is trapped and another antibody is allowed to react with the trapped antigen. In the ELISA, two kinds of antibody which recognizes the antigen to be detected or measured or the antibody fragment thereof in which antigen recognizing site is different are prepared and the first antibody or antibody fragments is previously adsorbed on a plate (such as a 96-well plate) and the second antibody or antibody fragment is labeled with a fluorescent substance such as FITC, an enzyme such as peroxidase, or biotin.

[0285] The plate to which the above antibody is adsorbed is allowed to react with the cell separated from living body or disrupted cell suspension thereof, tissue or disintegrated solution thereof, supernatant of cell culture, serum, pleural effusion, ascites, eye solution or the like, then allowed to react with a labeled monoclonal antibody or an antibody fragment and a detection reaction corresponding to the labeled substance is carried out. The antigen concentration in the sample to be tested can be calculated from a calibration curve prepared by a stepwise dilution of antigen of known concentration.

[0286] As antibody used for sandwich ELISA, any of polyclonal antibody and monoclonal antibody may be used or antibody fragments such as Fab, Fab' and $F(ab)_2$ may be used. As a combination of 2 kinds of antibodies used in sandwich ELISA, a combination of monoclonal antibodies or antibody fragments recognizing different epitopes may be used or a combination of polyclonal antibody with monoclonal antibody or antibody fragments may be used.

[0287] A fluorescent immunoassay includes a method described in the literatures [Monoclonal Antibodies - Principles and practice, Third Edition, Academic Press (1996*);* Manual for Monoclonal Antibody Experiments, Kodansha Scientific (1987)] and the like. As a label for the fluorescent immunoassay, any of known fluorescent labels [Fluorescent Immunoassay, by Akira Kawao, Soft Science, (1983)] may be used. Examples of the label include FITC, RITC and the like.

[0288] The luminescent immunoassay can be carried out using the methods described in the literature [Bioluminescence and Chemical Luminescence, Rinsho Kensa, 42, Hirokawa Shoten (1998)] and the like. As a label used for luminescent immunoassay, any of known luminescent labels can be included. Examples include acridinium ester, lophine or the like may be used.

[0289] Western blotting is a method in which an antigen or a cell expressing an antigen is fractionated by SDS (Sodium dodecyl sulfate)-PAGE [Antibodies-A Laboratory Manual (Cold Spring Harbor Laboratory, 1988)], the gel is blotted onto PVDF membrane or nitrocellulose membrane, the membrane is allowed to react with antigen-recognizing antibody or

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antibody fragment, further allowed to react with an anti-mouse IgG antibody or antibody fragment which is labeled with a fluorescent substance such as FITC, an enzyme label such as peroxidase, a biotin labeling, or the like, and the label is visualized to confirm the reaction. An example thereof is described below.

[0290] Cells or tissues in which a polypeptide having the amino acid sequence represented by SEQ ID NO: 53 is expressed are dissolved in a solution and, under reducing conditions, 0.1 to 30 μg as a protein amount per lane is electrophoresed by an SDS-PAGE method. The electrophoresed protein is transferred onto a PVDF membrane and allowed to react with PBS containing 1 to 10% of BSA (hereinafter referred to as "BSA-PBS") at room temperature for 30 minutes for blocking.

[0291] Here, the monoclonal antibody of the present invention is allowed to react therewith, washed with PBS containing 0.05 to 0.1% Tween 20 (hereinafter referred to as "Tween-PBS") and allowed to react with goat anti-mouse IgG labeled with peroxidase at room temperature for 2 hours. It is washed with Tween-PBS and a band to which the monoclonal antibody is bound is detected using ECL Western Blotting Detection Reagents (manufactured by Amersham) or the like to thereby detect a polypeptide having the amino acid sequence represented by SEQ ID NO: 1. As an antibody used for the detection in Western blotting, an antibody which can be bound to a polypeptide having no three-dimensional structure of a natural type is used.

[0292] The physicochemical method is specifically carried out by reacting TIM-3 as the antigen with the antibody or antibody fragment of the present invention to form an aggregate, and detecting this aggregate. Other examples of the physicochemical methods include a capillary method, a one-dimensional immunodiffusion method, an immunoturbidimetry, a latex immunoturbidimetry [Handbook of Clinical Test Methods, Kanehara Shuppan, (1988)] and the like.

[0293] For example, in a latex immunodiffusion method, a carrier such as polystyrene latex having a particle size of about of 0.1 to 1 μm sensitized with antibody or antigen may be used and when an antigen-antibody reaction is carried out using the corresponding antigen or antibody, scattered light in the reaction solution increases while transmitted light decreases. When such a change is detected as absorbance or integral sphere turbidity, it is possible to measure antigen concentration, etc. in the sample to be tested.

[0294] In addition, for the detection of the cell expressing TIM-3, known immunological detection methods can be used, and an immunoprecipitation method, an immuno cell staining method, an immune tissue staining method, a fluorescent antibody staining method and the like are preferably used.

[0295] An immunoprecipitation method is a method in which a cell expressing TIM-3 is allowed to react with the monoclonal antibody or antibody fragment of the present invention and then a carrier having specific binding ability to immunoglobulin such as protein G-Sepharose is added so that an antigen-antibody complex is precipitated. Also, the following method can be carried out. The above-described antibody or antibody fragment of the present invention is solid-phased on a 96-well plate for ELISA and then blocked with BSA-PBS.

[0296] When the antibody is in a non-purified state such as a culture supernatant of hybridoma cell, anti-mouse immunoglobulin or rat immunoglobulin or protein A or Protein G or the like is previously adsorbed on a 96-well plate for ELISA and blocked with BSA-PBS and a culture supernatant of hybridoma cell is dispensed thereto for binding. After BSA-PBS is discarded and the residue is sufficiently washed with PBS, reaction is carried out with a dissolved solution of cells or tissues expressing TIM-3. An immune precipitate is extracted from the well-washed plate with a sample buffer for SDS-PAGE and detected by the above-described Western blotting.

[0297] An immune cell staining method or an immune tissue staining method are a method where cells or tissues in which antigen is expressed are treated, if necessary, with a surfactant, methanol or the like to make an antibody easily permeate to the cells or tissues, then the monoclonal antibody of the present invention is allowed to react therewith, then further allowed to react with an anti-immunoglobulin antibody or binding fragment thereof subjected to fluorescent labeling such as FITC, enzyme label such as peroxidase or biotin labeling and the label is visualized and observed under a microscope.

[0298] In addition, cells can be detected by an immunofluorescent staining method where cells are allowed to react with a fluorescence-labeled antibody and analyzed by a flow cytometer [Monoclonal Antibodies - Principles and practice, Third Edition, Academic Press (1996), Manual for Experiments of Monoclonal Antibodies, Kodansha Scientific (1987)] in which cells are allowed to react with a fluorescence-labeled antibody and analyzed by a flow cytometer.

[0299] Particularly, the monoclonal antibody or antibody fragment of the present invention which binds to amino acids sequences of an extracellular region of the TIM-3 or a three-dimensional structure thereof can detect a cell expressing the polypeptide maintaining a natural three-dimensional structure by a fluorescence antibody method.

[0300] In addition, in the case of using FMAT8100HTS system (manufactured by Applied Biosystems) and the like among fluorescent antibody staining methods, the antigen quantity or antibody quantity can be measured without separating the formed antibody-antigen complex and the free antibody or antigen which is not concerned in the formation of the antibody-antigen complex.

The present invention can provide a monoclonal antibody or an antibody fragment of thereof, which binds to amino acids sequences of an extracellular region of TIM-3 or a three-dimensional structure and expresses ADCC activity; a hybridoma which produces the antibody; a DNA which encodes the antibody; a vector which comprises the DNA; a transformant

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obtained by transforming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a therapeutic agent or a diagnostic agent comprising the antibody or the antibody fragment thereof as an active ingredient.

[0301] Hereinafter, exemplary embodiments of the present invention will be described specifically. However, the present invention is not limited to the exemplary embodiments described below.

Example

[Example 1]

(Molecular cloning of human TIM-3 cDNA and establishment of stable expression cell)

[0302] Human TIM-3 cDNA was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and Human MTC panel (manufactured by Clontech) as a template. For primers, TIM-3 Fw2 (SEQ ID NO: 31) and TIM-3 Re2 (SEQ ID NO: 32) were used. The obtained PCR product was inserted into pGEM-T Easy vector (manufactured by Promega Corp.), and the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify. The plasmid DNA was extracted by the miniprep method.

[0303] By analyzing the sequence of purified DNA using primers TIM-3 Fw and TIM-3 Re2, the clone having the same sequence as the coding region of GenBank accession number NM_032782 was selected. A human TIM-3 retrovirus (hTIM-3/pMCs-IG) was constructed by recombination of the insert of the selected clone into pMCs-IRES-GFP vector (manufactured by Cell Biolabs Inc.) using Retrovirus Constructive System Ampho (manufactured by Takara Bio Inc.).

[0304] The human TIM-3 retrovirus was infected into Jurkat cell (ATCC accession NO: CRL-2063), EoL-1 cell (RIKEN CELL BANK NO: RCB0641) and L929 cell (manufactured by Sigma-Aldrich Co., LLC.) which were confirmed not to endogenously express TIM-3 was confirmed by flow cytometry analysis. After several passages, TIM-3 positive cells were collected by FACSAria (manufactured by BD Biosciences). Human TIM-3 stable expression cell line was established by recollecting the TIM-3 positive cells by FACSAria after several passages.

[Example 2]

(Preparation of expression vector of fusion protein of soluble extracellular human TIM-3-human Fc)

[0305] The cDNA encoding the extracellular region of human TIM-3 was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and hTIM-3/pMCs-Ig constructed in Example 1 as a template. For primers, pMCs-Fw (SEQ ID NO: 33) and TIM3ED-FcReXba 2 (SEQ ID NO: 34) were used.

[0306] The amplified PCR product was inserted into pTracer-CMV-FLAG-human Fc vetctor [modified vector in which FLAG and Fc domain of human IgG1 were inserted into pTracer-CMV (manufactured by Invitrogen) vector between *Xba*I site and *Apa*I site]. After the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify, the plasmid DNA (sTIM-3-FLAG-Fc/pTracerCMV) was extracted by miniprep method.

[0307] By the analysis of the DNA sequence using T7 (SEQ ID NO: 35) and hTIM-3 Fw1 (SEQ ID NO: 36) as a primer, the purified sTIM-3-FLAG-Fc/pTracerCMV was confirmed to have the same nucleotide sequence as the corresponding region in GenBank accession number NM_032782. The nucleotide sequence (from *EcoR*I recognition site to *Apa*I recognition site) was identical to the nucleotide sequence represented by SEQ ID NO: 37.

[Example 3]

(Preparation of expression vector of soluble extracellular human TIM-3)

[0308] The cDNA encoding the extracellular region of human TIM-3 was amplified by PCR using ExTaq (manufactured by Takara Bio Inc.) and sTIM-3-FLAG-Fc/pTracerCMV established in Example 2 as a template. As primers, TIM-3 Fw2 (SEQ ID NO: 31) and TIM3ED-FLAG4aa (SEQ ID NO: 39) were used.

[0309] Next, FLAG epitope was ligated by PCR method using ExTaq (manufactured by Takara Bio Inc.), TIM-3 Fw2 (SEQ ID NO: 31) and C-FLAG-NotR2 (SEQ ID NO: 40) as primers and the obtained PCR product as a template. The PCR product was inserted into pGEM-T Easy vector (manufactured by Promega Corp.). After the plasmid was introduced into TOP10 One shot competent cells (manufactured by Invitrogen) to amplify the plasmid DNA (sTIM-3-FLAG/pEF6Myc_HisC) was extracted by the miniprep method.

[0310] By the DNA sequence analysis using T7 (SEQ ID NO: 35) and BGH-R (SEQ ID NO: 41) as a primer, the purified sTIM-3-FLAG-Fc/pEF6 Myc_HisC was confirmed to have the same nucleotide sequence as the corresponding region in GenBank accession number NM_032782.

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[Example 4]

(Preparation of the fusion protein of soluble extracellular human TIM-3-human Fc and soluble extracellular human TIM-3)

[0311] Each of sTIM-3-FLAG-Fc/pTracerCMV plasmid DNA and sTIM-3-FLAG-Fc/pEF6 Myc_HisC plasmid DNA obtained in Example 2 and Example 3, respectively, was introduced into HEK293F cells (manufactured by Invitrogen) and was transiently expressed. Six days after, each of the cell supernatant was recovered and used for protein purification.

[0312] The culture supernatant containing the fusion protein of soluble extracellular human TIM-3-human Fc or soluble extracellular human TIM-3 was recovered by centrifugation six days after transfection. The fusion proteins were purified by an anti-FLAG column prepared using Anti-FLAG M2 Agarose Affinity gel (manufactured by Sigma-Aldrich Co., LLC.) and by FLAG peptides (manufactured by Sigma-Aldrich Co., LLC.) according to the manufacturer's protocols.

[0313] The eluted samples were fractionated and then each fraction was analyzed by SDS-PAGE under reduced conditions, and then silver staining and Western blotting were carried out. For Western blotting, an anti-FLAG M2 antibody (manufactured by Sigma-Aldrich Co., LLC.) and an alkaline phosphatase labeled rabbit anti-mouse immunoglobulin antibody was used (manufactured by Dako). The fraction containing the target protein was concentrated using Amicon Ultra 4 10K (manufactured by Millipore) and was applied for gel filtration chromatography using Superdex 200 gp column (manufactured by GE Healthcare).

[0314] After fractionation, each fraction was applied to SDS-PAGE under reduced condition, silver stained and then Western blotted. The fraction in which the target protein was found was concentrated and washed with 0.5 ml of PBS. The soluble extracellular human TIM-3-human Fc fusion protein or soluble extracellular human TIM-3 was collected after filtering through the MILLEX-GV sterilizing filter (manufactured by Millipore) having a pore diameter of 0.22 μm. The purity of the protein was measured by Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) which is a kit to detect endotoxin and confirmed that the fraction was sufficiently purified.

[Example 5]

(Preparation of human antibody expressing mouse)

[0315] The mice used for immunization has a genetic background in which both of endogenous Ig heavy chain locus and κ light chain locus were homozygously disrupted and maintained a chromosome 14 fragment (SC20) comprising a human heavy chain transchromosome, and a human Igκ chain transgene (KCo5) at the same time. This mouse strain was established by cross breeding mice strain A which had human Ig heavy chain loci and mice strain B which maintained human Igκ chain transgene.

[0316] Mouse strain A is a homozygote in which both of the endogenous Ig heavy chain locus and κ light chain locus are homozygously disrupted, and maintains a chromosome 14 fragment (SC20) which can be transmitted to the progeny and for example, described in Tomizuka *et al.* (Tomizuka, K. et al., 2000, Proc. Natl. Acad. Sci. U.S.A. 97:722-727).

[0317] Mouse Strain B is a mouse strain (transgenic mouse) in which both of the endogenous Ig heavy chain locus and κ light chain locus are homozygously disrupted, and maintains a human Igκ light chain transgene (KCo5), and is ,for example, described in Fishwild et al. (1996) [Nature Biotechnology 14:845-851].

[0318] The animal individual was obtained by cross-breeding male mouse of strain A and female mouse of strain B, or female mouse of strain A and male mouse of strain B and was a mouse in which human Ig heavy chain and Igκ light chain could be detected at the same time in their serum [Ishida& Lonberg, IBC's 11th Antibody Engineering, Abstract 2000] was further used for immunological experiments. For your information,, the human antibody expressing mouse (hereinafter referred to as "KM mouse") can be obtained from Kyowa Hakko Kirin by establishing a license.

[Example 6]

(Preparation of anti-human TIM-3 monoclonal antibody)

[0319] The preparation of monoclonal antibody in the present Example was carried out according to the general methods, such as published in Monoclonal Antibody Experiment Manual, (Yasuhigashi Tamie et al., Kodansha, 1991). Either TIM-3 expressing L929 cell prepared in Example 1 or the soluble extracellular human TIM-3-human Fc fusion protein prepared in Example 4 was used as a TIM-3 immunogen. The immunized animal was KM mouse described above.

[0320] The TIM-3 expressing L929 cell was intraperitoneally injected into mice at a dose of 1×10^7 cells/animal. After the first immunization, the same cell was further administered for another three times or more thereafter. Three days before obataining the spleen, the soluble extracellular human TIM-3-human Fc fusion protein prepared in Example 4 was administered from caudal vein at 20 μg/mouse. The spleen was surgically removed from the immunized mouse, put into 4 ml of PBS and mashed on a mesh (cell strainer: manufactured by Falcon) using a syringe piston.

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[0321] The cells were precipitated by centrifuging the cell suspension which was passed through the mesh, and the cells were resupsended in 1 ml of Red Blood Cell Lysis Buffer (manufactured by Sigma-Aldrich Co., LLC.). After incubating for 5 min at room temperature, 10 ml of serum free DMEM medium (manufactured by Invitrogen) (hereinafter referred to as "serum free DMEM medium") containing 50 unit/ml penicillin and 50 μg/ml streptomycin was added, and the cells were precipitated by centrifugation.

[0322] The cell pellets were resuspended in serum free DMEM medium. On the other hand, myeloma cell line SP2/0 (ATCC NO: CRL-1581) was cultured in DMEM medium containing 10% FBS, 50 unit/ml penicillin and 50 μg/ml streptomycin (hereinafter referred to as "DMEM medium containing serum") and incubated at 37°C under 5% CO_2 so as not exceed the cell number of 1×10^6 cells/ml.

[0323] The SP2/0 cells were washed with 10 ml of serum free DMEM medium and suspended in serum free DMEM medium as in the case of the spleen derived cells. These suspension of spleen derived cells and suspension of myeloma cell were mixed at a ratio of 5:1, centrifuged, and the supernatant was completely removed. To fuse the cells, 1 ml of 50% (w/v) of polyethylene glycol (manufactured by Boehringer Mannheim Corp.) was slowly added while stirring the pellet with the pipette tip, and then 1 ml of serum free DMEM medium preheated at 37°C was added. Slowly, 5 ml and 10 ml of serum free DMEM was added followed by 5 min incubation at 37°C under 5% CO_2.

[0324] After the centrifugation and removal of supernatant, the fused cells were resuspended in 50 ml of DMEM medium containing 10% FBS (manufactured by Invitrogen), penicillin-streptomycin-glutamine (manufactured by Invitrogen, 100-fold dilution of Catalog No.10378-016), IL-6 (5 ng/ml), and 2-mercaptoethanol (manufactured by Invitrogen, 1000-fold dilution of Catalog No. 21985-023) (hereinafter referred to as "DMEM medium containing IL-6") and incubated at 37°C under 5% CO_2.

[0325] The next day, cells were harvested by pipetting, centrifuged and the pellet was resuspended in 10 ml of DMEM medium containing IL-6. On the next day, Hypoxanthine-aminopterin-thymidine (hereinafter referred to as "HAT", manufactured by Sigma-Aldrich Co., LLC.) was added. After incubating for about 7 to 10 days, the culture supernatant was collected for hybridoma screening.

[Example 7]

(Preparation of anti-human TIM-3 mouse monoclonal antibody)

[0326] The preparation of monoclonal antibody in the present Example was carried out according to the general methods published in Introduction to Monoclonal Antibody Experiment (Yasuhigashi Tamie et al., Kodansha, 1991). Either TIM-3 expressing L929 cell prepared in Example 1 or soluble extracellular human TIM-3 prepared in Example 4 was used as a TIM-3 immunogen. The immunized animal was Balb/c mouse (purchased from Nihon Charles River).

[0327] First, a Balb/c mouse was immunized by injecting TIM-3 expressing L929 cells intraperitoneallyat a dose of 1×10^7 cells/animal. After the first immunization, the same cell was further administered for another three times or more thereafter. Three days before extracting the spleen, the soluble extracellular human TIM-3 protein prepared in Example 4 was administered from caudal vein at 20 μg/mouse. The spleen was surgically removed from the immunized mouse, put into 4 ml of PBS and mashed on a mesh (cell strainer: manufactured by Falcon) using a syringe piston.

[0328] The cells were precipitated by centrifuging the cell suspension which was passed through the mesh, and the cells were resuspended in 1 ml of Red Blood Cell Lysis Buffer (manufactured by Sigma-Aldrich Co., LLC.). After incubating for 5 min at room temperature, 10 ml of serum free DMEM medium was added, and then cells were precipitated by centrifugation. The pellets were resuspended in serum free DMEM medium. On the other hand, myeloma cell line SP2/0 (ATCC NO: CRL-1581) were cultured in DMEM medium by incubating at 37°C under 5% CO_2 to the cell number of 1×10^6 cells/ml or less.

[0329] Similar to the spleen derived cells, the SP2/0 cells were washed with 10 ml of serum free DMEM medium and suspended in serum free DMEM medium. The suspension of spleen cell and the suspension of myeloma cell were mixed at a ratio of 5:1. After centrifugation, the supernatant was removed. To this pellet, 1 ml of 50% (w/v) of polyethylene glycol (manufactured by Boehringer Mannheim Corp.) was slowly added while stirring the pellet with the pipette tip, and then 1 ml of serum free DMEM medium preheated to 37°C was added.

[0330] To this suspension, 5 ml of DMEM medium was slowly added. After further adding 10 ml of serum free DMEM, the suspension was incubated for 5 min at 37°C under 5% CO_2. After the suspension was centrifuged, the cell pellets were resuspended in 50 ml of DMEM medium containing IL-6 and cultured at 37°C under 5% CO_2. After incubation for one day, cells were collected by pipetting. After centrifugation, the pellet was resuspended in 10 ml of DMEM medium containing IL-6. On the next day, HAT medium (manufactured by Sigma-Aldrich Co., LLC.) was added. After incubation for about 7 to 10 days, the culture supernatant was recovered and used for hybridoma screening.

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[Example 8]

(Screening of hybridoma producing a human or mouse monoclonal antibody which binds to human TIM-3)

[0331] The hybridoma was screened using the cell supernatant prepared in Example 6 and Example 7. The method to be used was a flow cytometry method using human TIM-3 expressing cell lines. First, the human TIM-3 expressing Jurkat cell or EoL-1 cell prepared in Example 1 were washed with staining medium (PBS containing 2% FBS and 0.05% sodium azide), and then resuspended in 1 ml of staining medium to give a cell density of 1×10^6 cells/ml. The cell suspension was dispensed at 10 μl/well into a 96-well culture plate.

[0332] Then, 50 μl of the hybridoma supernatant was added and incubated for 30 min at 4°C. After the cells were washed twice with the staining medium, 50 μl of labeled antibody which was diluted 200-fold with staining medium was added to each well and incubated for 30 min at 4°C. As for labeled antibodies, Goat $F(ab')_2$ Anti-Human IgG (γ chain specific)-R-PE (manufactured by SouthernBiotech) were used for the human monoclonal antibody and Goat $F(ab')_2$ Anti-Mouse IgG(H+L)R-PE (manufactured by SouthernBiotech) was used for the mouse monoclonal antibody.

[0333] After washing twice with staining medium, the cells were analysed using FACSCalibur (manufactured by BD Biosciences) as a primary screening. After positive clones were picked and expanded, the hybridoma cells were clone-sorted by FACSAria (manufactured by BD Biosciences) and cultured for seven days in DMEM medium containing IL-6 which further contains Hypoxanthine-thymidine (hereinafter referred to as "HT", manufactured by Sigma-Aldrich Co., LLC.).

[0334] Harvested supernatants were screened as the same as in the primary screening method for cloning of hybridomas expressing anti-human TIM-3 human monoclonal antibody and hybridomas expressing anti-human TIM-3 mouse monoclonal antibody and selected hybridoma clone was used for purification of monoclonal antibody.

[Example 9]

(Purification of hybridoma derived monoclonal antibody)

[0335] An anti-TIM-3 human or mouse monoclonal antibody was purified from the anti-human TIM-3 human or mouse monoclonal antibody expressing hybridoma prepared in Example 8. Briefly, hybridoma supernatant containing a high concentration of the anti-TIM-3 antibody was prepared using a CELLine antibody production system (manufactured by BD Biosciences) and the anti-human TIM-3 monoclonal antibody was purified from the supernatant. First, to adapt the cloned hybridoma to the serum free medium, the hybridomas were cultured for several days in a medium mixing DMEM medium containing HT and IL-6 and BD Cell MAb serum free medium (manufactured by BD Biosciences) at a ratio of 1:1, and then cultured for several days in a medium having a mixing ratio of 1:2.

[0336] Next, the hybridomas were cultured in a BD Cell MAb serum free medium (manufactured by BD Biosciences) and adapted hybridomas to the serum free medium. The hybridoma cells adapted to the serum free medium were cultured in CL-1000 flasks according to the manufacturer's instructions and the supernatant of the hybridoma containing high concentration of the anti-TIM-3 antibody was collected from the flasks. The antibody from the supernatant of the hybridoma was purified in accordance with the standard procedures using Protein A.

[0337] Specifically, MabSelect (manufactured by GE Healthcare) were packed in an open column and the supernatant diluted 2-fold by PBS was loaded and then eluted with 0.1 mol/L Glycine-HCl (pH 2.7), followed by concentration with Amicon Ultra (manufactured by Millipore). Next, by using a NAP-5 column (manufactured by GE Healthcare), the obtained solution was equilibrated with PBS buffer, and then sterilized by filtration to obtain 4 clones of the anti-human TIM-3 human monoclonal antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) and one clone (antibody 8213) of the anti-human TIM-3 mouse monoclonal antibody.

[Example 10]

(Identification of isotype of anti-TIM-3 human monoclonal antibody)

[0338] Each of the isotype of anti-TIM-3 human monoclonal antibodies which was prepared in Example 9 was determined by a solid-phase ELISA and flow cytometry.
Specifically, as for the solid-phase ELISA, soluble extracellular human TIM-3 obtained by Example 4 was diluted with a Carbonate-Biacarbonate Buffer (manufactured by Sigma-Aldrich Co., LLC.) to give a concentration of 1 μg/ml and dispensed at 50 μl/well into a 96-well culture plate (Maxisorp, manufactured by Nunc). The soluble extracellular human TIM-3 was adsorbed to the microplate by incubating overnight at 4°C.

[0339] After the supernatant was discarded, SuperBlock Blocking Buffer in TBS (manufactured by PIERCE) was added to the plate and then incubated for 10 min at room temperature and 50 μl of the culture supernatant of the hybridoma

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was added and then incubated for 30 min at room temperature. After washing each well with Tris-buffered saline containing 0.1% Tween20 (TBS-T), 50 μl of each of HRP-labeled mouse anti-human IgG1, anti-human IgG2, anti-human IgG3 or anti-human IgG4 antibodies, each antibody was diluted 2000, 2000, 2000 and 4000-fold with TBS-T containing 10% SuperBlock Blocking Buffer, respectively; manufactured by SouthernBiotech), were added and incubated for 30 minutes at room temperature.

[0340] After washing each well with TBS-T, 50 μl of substrate buffer (TMB, manufactured by DAKO) was added and then incubated for 20 min at room temperature. The reaction was stopped by adding 50 μl of 0.5 mol/L sulfuric acid (manufactured by Wako Pure Chemical Industries, Ltd.). The absorbance at wavelength of 450 nm (reference wavelength: 570 nm) was measured on a microplate reader (VersaMax, manufactured by Molecular Devices, LLC.).

[0341] In addition, in flow cytometry method, the Jurkat cell which expressed human TIM-3 prepared in Example 1 was washed with staining medium, and then 50 μl of the supernatant of the hybridoma was added and incubated for 30 min at 4°C. After washing, 50 μl of each of Mouse anti-Human IgG1-PE, Mouse anti-Human IgG2-PE, Mouse anti-Human IgG3 (Hinge)-PE and Mouse anti-Human IgG4(Fc)-PE (manufactured by SouthernBiotech) which was diluted 100-fold with staining medium was added, allowed to stand still for 30 min at 4°C and then analyzed using FACSCalibur flow cytometer (manufactured by BD Biosciences).

[0342] Based on the result, the isotypes of antibody 512, antibody 644, and antibody 4545 were IgG1, IgG4 and IgG2, respectively.

[Example 11]

(Identification of the isotype of the mouse monoclonal antibody for human TIM-3)

[0343] The isotype of the anti-human TIM-3 mouse monoclonal antibody prepared in Example 9 was determined using Iso Strip Mouse Monoclonal Antibody Isotyping Kit (manufactured by Roche-diagnostics). By using the supernatant of the hybridoma containing the anti-TIM-3 antibody prepared in Example 8, the isotype was determined according to the manufacturer's instructions. As a result, the isotype of antibody 8213 was determined as IgG2b.

[Example 12]

(Isolation of anti-TIM-3 monoclonal antibody gene)

[0344] The gene of anti TIM-3 human or mouse monoclonal antibody which was prepared in Example 9 was isolated from the hybridoma which produced anti-human TIM-3 human or mouse monoclonal antibody prepared in Example 9. Total RNA was extracted from the cloned hybridoma using High Pure RNA Isolation Kit (manufactured by Roche-Diagnostics), according to the manufacturer's instructions. The cloning of the cDNA encoding the variable region was carried out using SMART RACE cDNA amplification Kit (manufactured by Clontech) in accordance with the instructions attached thereto.

[0345] The heavy chain (VH) of the human monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hh-6 (SEQ ID NO: 44). Using part of the reaction product as a template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hh-3 (SEQ ID NO: 45). The cloning of the PCR product was carried out using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R primer) and the amino acid sequence was deduced from the nucleotide sequence.

[0346] As a result, the nucleotide sequence and the amino acid sequence of VH of antibody 512 were represented by SEQ ID NO: 54, was and SEQ ID NO: 55, respectively, the nucleotide sequence and the amino acid sequence of VH of antibody 644 were represented by SEQ ID NO: 58 and SEQ ID NO: 59, respectively, the nucleotide sequence and the amino acid sequence of VH of antibody 4545 were represented by SEQ ID NO: 7 and SEQ ID NO: 8, respectively, and the nucleotide sequence and the amino acid sequence of VH of antibody 4177 were represented by SEQ ID NO: 17 and SEQ ID NO: 18, respectively. Each of the obtained variable regions obtained and the constant region of human IgG1 were inserted and ligated into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0347] Human monoclonal antibody light chain (VL) was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hk-2 (SEQ ID NO: 46). Using part of the reaction product as a template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer hk-6 (SEQ ID NO: 47). The PCR product was cloned using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0348] As a result, the nucleotide sequence and the amino acid sequence of VL of antibody 512 were represented by SEQ ID NO: 56 and SEQ ID NO: 57, respectively, the nucleotide sequence and the amino acid sequence of VL of

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antibody 644 were represented by SEQ ID NO: 60 and SEQ ID NO: 61, respectively, the nucleotide sequence and the amino acid sequence of VL of antibody 4545 were represented by SEQ ID NO: 9 and SEQ ID NO: 10, respectively, and the nucleotide sequence and the amino acid sequence of VH of antibody 4177 were represented by SEQ ID NO: 19 and SEQ ID NO: 20, respectively. Each of the obtained variable regions and constant region of κ chain were inserted and ligated into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0349] The heavy chain (VH) of mouse monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and a primer, mH_Rv1 (SEQ ID NO: 48). Using part of the reaction product e as template, PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mH_Rv2 (SEQ ID NO: 49).

[0350] The cloning of the PCR product was carried out using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). The nucleotide sequence was analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0351] As a result, the nucleotide sequence and the amino acid sequence of VH of antibody 8213 were represented by SEQ ID NO: 27 and SEQ ID NO: 28, respectively. The obtained variable region and the constant region of mouse IgG2a were ligated and inserted into the expression vector, N5KG1 (manufactured by Biogen IDEC Inc.).

[0352] The light chain (VL) of the mouse monoclonal antibody was amplified by PCR using UPM (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mK_Rv1 (SEQ ID NO: 50). Additionally, part of the reaction product was used as a template, and PCR was carried out using NUP (SMART RACE cDNA amplification Kit; manufactured by Clontech) and primer mK_Rv2 (SEQ ID NO: 51). The PCR product was cloned using Zero Blunt TOPO PCR Cloning Kit (manufactured by Invitrogen). Nucleotide sequences were analyzed by universal primers of the vector (e.g., T7 or M13R) and the amino acid sequence was deduced from the nucleotide sequence.

[0353] As a result, the nucleotide sequence and the amino acid sequence of the VL of antibody 8213 were represented by SEQ ID NO: 29 and SEQ ID NO: 30, respectively. The variable region obtained and the constant region of κ chain were ligated and inserted into the above mentioned expression vector encoding VH of antibody 8213.

[0354] In addition, in order to obtain anti-dinitrophenyl (DNP) human IgG1 antibody as a negative control, the nucleotide sequence of the heavy chain (VH) of the antibody (SEQ ID NO: 62), the nucleotide sequence of the light chain of the antibody (SEQ ID NO: 64), and the constant region of human IgG1 and the κ chain were inserted into expression vector N5KG1 (manufactured by Biogen IDEC Inc.).

[Example 13]

(Purification of recombinant anti-TIM-3 human monoclonal defucose antibody)

[0355] A cell expressing a recombinant antibody was produced by introducing the recombinant antibody expression vector prepared in Example 12 into the host cell. As the host cell for expression, $FUT8^{-/-}$CHO cells were used in accordance with a published report *(Clin Cancer Res* 2006: 12(9) Iida *et al.*). Briefly, the expression vectors for each of 512, 644, 4545 and 4177 prepared in Example 12 were introduced into $FUT8^{-/-}$ CHO cell using FreeStyle™ MAX Reagent (manufactured by Invitrogen). The $FUT8^{-/-}$CHO cells were cultured on a shaker at 37°C under 5% CO_2. The culture supernatant was recovered after about five days.

[0356] The recovered supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. Antibodies were affinity purified using PBS as the binding buffer and 0.02 mol/L sodium citrate (pH 2.7, 50 mmol/L NaCl) buffer as the elution buffer. The elution fraction was added with 0.2 mol/L of sodium phosphate buffer (pH 7.0).

[0357] The prepared antibody solution was equilibrated with PBS using NAP-25 column (manufactured by GE Healthcare) and concentrated with Amicon Ultra (10000 cut, manufactured by Millipore). After sterilization by filtration, the recombinant anti-TIM-3 human monoclonal defucose antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) were obtained. The purity of the protein was determined by Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that these antibodies were sufficiently purified.

The vector for expressing anti-DNP prepared in Example 12 was introduced into CHO-DG44 cells. The CHO-DG44 cells were cultured on a shaker at 37°C under 5% CO_2. The cultured supernatant was recovered following incubation for 5 days. The collected supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. The affinity purification of the antibodies was carried out using PBS as the binding buffer and 0.02 mol/L of sodium citrate (pH 2.7, 50 mmol/L NaCl) buffer as the elution buffer. The elution fraction was added with 0.2 mol/L of sodium phosphate buffer (pH 7.0). The prepared antibody solution was equilibrated with PBS and concentrated using NAP-25 column (manufactured by GE Healthcare) with Amicon Ultra (10000 cut, manufactured by Millipore) respectively. After sterilization by filtration, the recombinant anti-DNP antibody was obtained. The purity of the protein was determined using a Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that the antibody was sufficiently purified.

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[Example 14]

(Purification of recombinant anti-TIM-3 mouse monoclonal antibody)

[0358] The vector for expressing antibody 8213 prepared in Example 12 was introduced into HEK293F cells (manufactured by Invitrogen) using 293 fectin (manufactured by Invitrogen). HEK293F cells were incubated on a shaker at 37°C under 5% CO_2. The cultured supernatant was collected after incubation for five days. The collected supernatant was purified using Protein A (manufactured by GE Healthcare) and further using 0.8×40 cm column (manufactured by BioRad) and the like, depending on the sample amount. The affinity purification of the antibody was carried out using PBS as a binding buffer and 0.02 mol/L sodium citrate buffer (pH 2.7, 50 mmol/L NaCl) as an elution buffer.

[0359] To the elution fraction, 0.2 mol/L of sodium phosphate buffer (pH 7.0) was added. The prepared antibody solution was equilibrated with PBS using NAP-25 column (manufactured by GE Healthcare) and concentrated with Amicon Ultra (10000 cut, manufactured by Millipore). After sterilization by filtration, the recombinant anti-TIM-3 mouse monoclonal antibody (8213 antibody) was obtained. The purity of the protein was determined using a Limulus ES-II kit Wako (manufactured by Wako Pure Chemical Industries, Ltd.) and found that the antibody was sufficiently purified.

[Example 15]

(Fluorescent labeling of purified monoclonal antibody)

[0360] The fluorescent labeling of the hybridoma derived monoclonal antibody and recombinant monoclonal antibody was carried out by using Alexa Fluor 647 Monoclonal Antibody Labeling Kit (manufactured by Invitrogen) according to the manufacturer's instructions. After the labeling, each antibody was confirmed to be positively labeled with Alexa Fluor 647 (hereinafter referred to as "Alexa-647") by FACSCalibur (manufactured by BD Biosciences) analysis for TIM-3 expressing cells.

[Example 16]

(Classification of epitopes using competition test-1)

[0361] The relation of epitopes between antibodies prepared in Example 13 and Example 14, and commercially available anti-TIM-3 antibody 344823 (Clone 344823, manufactured by R&D Systems) was analyzed by a competition test. In brief, the test antibody was allowed to interact with the TIM-3 expression cell prepared in Example 1, and then whether another anti-TIM-3 antibody could further bind to TIM-3 expressing cells was evaluated by flow cytometry method.

[0362] In the first step, whether or not the competition between each of the monoclonal antibodies prepared in Example 13 and Example 14 (antibody 512, antibody 644, antibody 4177 and antibody 8213) and antibody 34823 were examined. First, human TIM-3 expressing EoL-1 cells and the parent cell, EoL-1 cells were washed with staining medium and then allowed to react with each of the purified subjective monoclonal antibodies (antibody 512, antibody 644, antibody 4177 and antibody 8213) (4°C, 30 min). The final concentration was 10 μg/ml. Next, the PE labeled antibody 344823 (Clone 344823, manufactured by R&D Systems) was added and allowed to react (4°C, 30 min). After washing with staining medium, 7-ADD (manufactured by BD Biosciences) was added and analyzed using FACSCalibur (manufactured by BD Biosciences).

[0363] As a result, the goat derived anti-human TIM-3 polyclonal antibody (manufactured by R&D Systems) used as a positive control and antibody 644 almost completely blocked the binding of PE-labeled antibody 344823 to TIM-3 expressing cell. However, antibody 512, 4545 and 8213 did not inhibit the binding of PE-labeled antibody 344823 to TIM-3 expressing cell. Antibody 4177 inhibited the binding of PE-labeled antibody 344823 to TIM-3 expressing cells, but this inhibition was weaker than that of antibody 644. Accordingly, antibody 644 competed with antibody 344823, but antibodies 512, 4545 and 8213 did not compete with antibody 344823. It was found that the antibody 4177 and antibody 344823 competed with each other, but they competed partially.

[0364] In the second step, among the anti-TIM-3 monoclonal antibodies tested, antibodies 512, 4545 and 8213 which did not interact with antibody 344823 were examined whether or not they competed. The experiment was carried out in the same manner as the first step except for using Alexa-647 labeled antibody 512 or Alexa-647 labeled antibody 8213 prepared in Example 15 as a labeled antibody. As a result, antibody 4545 and antibody 8213 did not inhibit the binding of Alexa-647 labeled antibody 512 to the TIM-3 expressing cells.

[0365] However, antibody 4545 inhibited the binding of Alexa-647 labeled antibody 8213 to TIM-3 expressing cells. Therefore, it was found that antibody 4545 and antibody 8213 competed with each other, but these two antibodies did not compete with antibody 512. This result suggested that antibody 512 recognized different epitope from antibody 344823 and antibody 8213.

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[0366] In the third step, a competitive assay with antibody 512 and antibody 8213 was carried out on antibody 4177 which was shown to partially compete with antibody 344823 in the first step. The experiment was performed in the same manner as described in second step. As a result, antibody 4177 did not inhibit the binding of the Alexa-647 labeled antibody 512 to the TIM-3 expressing cells, but inhibited the binding of the Alexa-647 labeled antibody 8213 to the TIM-3 expressing cells. Therefore, it was found that antibody 4177 did not compete with antibody 512, but only with antibody 8213.

[0367] Based on these results, among the 5 antibodies prepared in Example 13 and Example 14, antibody 644 recognized an epitope which was near the epitope to which antibody 344823 recognized; antibody 512 recognized an epitope different from of the epitope to which antibody 344823 and other four antibodies (antibody 644, antibody 4545, antibody 4177 and antibody 8213) recognized.

[0368] It was suggested that antibody 8213 recognized epitope different from of the epitope to which antibodies 344823 or 512 recognized. It was suggested that antibody 4545 recognized an epitope which was near the epitope to which antibody 8213 recognized. However, antibody 4177 recognized the epitope which was near the epitope to which antibody 8213 recognized, and also weakly recognized an epitope which was near the epitope to which antibody 344823 recognized.

[Example 17]

(Antibody dependent cellular cytotoxicity test of recombinant anti-TIM-3 human monoclonal defucose antibody)

[0369] Regarding the cellular cytotoxic activity via antibody, the ADCC activity against the target cell was measured by using human Peripheral Blood Mononuclear Cells (hereinafter referred to as "PBMC") as an effector in the presence of the antibody. First, peripheral blood was collected from the healthy human donor and mixed with anticoagulant. The obtained blood was loaded onto Ficoll-Plaque Plus (manufactured by GE Healthcare), and centrifuged at 2000 rpm for 20 min using a large scale centrifuge (CF9RX, manufactured by Hitachi Koki Co., Ltd.) so as not to disturb the boundary.

[0370] The middle fraction containing cells were collected and washed with PBS, and then centrifuged at 900 rpm for 20 min to remove the platelets. Thus obtained PBMC was used for the measurement. Next, the Daudi cell used as a target cell and the PBMC were mixed at the ratio of effector/target = 50.

[0371] Each of the recombinant anti-human monoclonal defucose antibodies (antibody 512, antibody 644, antibody 4545 and antibody 4177) prepared in Example 13 and Example 14, and anti-DNP antibody as a human IgG1 control was suspended in the medium to give a final concentration of 1 μg/ml and a total volume of 100 μl. After mixing, the solution was incubated at 37°C under 5% CO_2 for 4 hr. The lysis ratio of the target cell was evaluated based on the amount of LDH released into the medium. The LDH activity and lysis ratio were calculated using CytoTox 96 Non-Radioactive Cytotoxicity Assay (manufactured by Promega Corp.) in accordance with the instructions attached thereto. As statistical analysis, standard Student's t-test was used wherein a sample having a risk rate (p) of less than 0.05 was considered as one which was significantly different.

[0372] As the result that the ADCC activity on Daudi cells was measured, it was found that antibody 4545 and antibody 4177 exhibited the significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody. This result suggests that anti-TIM-3 mouse antibody 8213 and anti-TIM-3 antibody which competes with anti-TIM-3 mouse antibody 8213 have high ADCC activity.

[0373] Similarly, the ADCC activity was measured by using recombinant anti-TIM-3 human monoclonal defucose antibodies (antibody 4545 and antibody 4177) prepared in Example 13, commercially available anti-TIM-3 monoclonal antibody 344823, and anti-DNP antibody as a negative control.

[0374] First, peripheral blood was collected from the healthy human donor and mixed with anticoagulant. The obtained blood was then loaded onto Ficoll-Plaque Plus (manufactured by GE Healthcare), and centrifuged at 2000 rpm for 20 min using a large scale centrifuge (CF9RX, manufactured by Hitachi Koki Co., Ltd.) so as not to disturb the boundary. The middle fraction containing cells were collected and washed with PBS, and then centrifuged at 900 rpm for 20 min to remove the platelets. Thus obtained PBMC fraction was used for the measurement of t the ADCC activity.

[0375] Next, the Daudi cell used as a target cell and the PBMC were mixed at the ratio of effector/target = 25. Each of the antibody 4545, antibody 4177, antibody 344823, anti-DNP antibody and PBS were suspended in the medium to give a final concentration of 1 μg/ml and a total volume of 100 μl. After mixing, the solution was incubated at 37°C under 5% CO_2 for 4 hr. The lysis ratio of the target cell was evaluated based on using the amount of LDH released into the medium.

[0376] The LDH activity and the lysis ratio were calculated using CytoTox 96 Non-Radioactive Cytotoxicity Assay (manufactured by Promega Corp.) in accordance with the instructions attached thereto. As statistical analysis, standard Student's t-test was used wherein a sample having a risk rate (p) of less than 0.05 was considered as one which was significantly different. As the result of the ADCC activity on Daudi cells was measured, it was found that antibody 4545 and antibody 4177 exhibited the significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody.

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On the other hand, antibody 344823 exhibited no significant increase in the lysis ratio of the target cell as compared to anti-DNP antibody and PBS.

[Example 18]

(Calculating the bound/dissociation constant using anti-TIM-3 antibody)

[0377] The bound/dissociation constant was analyzed using surface plasmon resonance based biosensor (Biacore, manufactured by GE Healthcare). In brief, an anti-human antibody or an anti-mouse antibody was immobilized on the CM5 sensor chips. Next, the anti-TIM-3 human or mouse antibody were made to flow to bind the chip, and then soluble extracellular human TIM-3 prepared in Example 4 was made to flow to examine the bound/association constant of TIM-3 to the anti-TIM-3 antibody. Throughout the experimental process, basically the experimental method provided by GE Healthcare for calculating bound/dissociation constant was used.

[0378] Specifically, CM5 was used as the sensor chip (Research grade, manufactured by GE Healthcare). First, CM5 chip was activated by flowing through a mixed solution of equal parts of 400 mmol/L EDC (N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrocholoride) and 100 mmol/L NHS(N-hyderoxysuccinimide). Next, the anti-human antibody attached to the Human Antibody Capture Kit (manufactured by GE Healthcare) was diluted with the solution attached to the kit and the obtained solution was made to flow so that the required amount of the antibody to an antibody for a human antibody was immobilized on the CM5 chip.

[0379] For the mouse antibody, the antibody against mouse antibody was immobilezed on the CM5 surface by diluting in the solution, which was provided in the Mouse Antibody Capture Kit (manufactured by GE Healthcare antibody). The activated chip surface was blocked by the passing through 1 mol/L for ethanolamide hydrochloride and inactivated.

[0380] Next, one kind of the anti-TIM-3 antibodies was diluted with HBS-EP buffer (manufactured by GE Healthcare) per one flow cell and passed through so as to bind the antibody for the human antibody or antibody for the mouse antibody immobilized on the chip surface. Then soluble extracellular human TIM-3 was flown through. To dissociate the bound anti-TIM-3 antibody from the soluble extracellular human TIM-3, either the total volume of 3 mol/L of $MgCl_2$ which was attached to the Human Antibody Capture Kit or Glycine-HCl (pH 1.7) which was attached to the Mouse Antibody Capture Kit were flown through the cell.

[0381] The above described procedures were considered as one step, and the similar procedure was repeated using different concentrations of the soluble extracellular human TIM-3, to collect data (sensorgram) for calculating the bound/association constant. The concentration of soluble extracellular human TIM-3 used as an analyte was calculated by measuring the absorbance at 280 nm and then converting the value of 1.4 OD to 1 mg/mL.

[0382] The molecular weight of the soluble extracellular human TIM-3 was calculated as 42.8 kDa based on the mobility on electrophoresis. The data was analyzed using Biaevaluation soft-ware (manufactured by GE Healthcare) according to the Biaevaluation Software Handbook. Specifically, the simultaneous Kinetics analysis was performed and the association rate constant (Ka) and dissociation rate constant (Kd) was calculated, by basically adopting a 1:1 Langmuir Binding reaction model was used for fitting. Then the value of the dissociation constant (K_D) was calculated from Kd/Ka.

[0383] Most of the anti-human TIM-3 human monoclonal antibody had the K_D value of the order of 10^{-9} mol/L. In this aspect, there was no correlation between ADCC activity and binding affinity targeting for TIM-3.

[0384] When the anti-human TIM-3 monoclonal antibody was monitored (e.g., monitored the dissociation state for 30 min), there was no dissociation detected with the antigen soluble extracellular human TIM-3 as an antigen. As a result, it was found that it was possible to produce a monoclonal antibody having a very high affinity for human TIM-3.

[Example 19]

(Classification of epitopes using competition test-2)

[0385] The epitopes of anti-TIM-3 antibodies, i.e., antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823, antibody F38-2E2 and anti-DNP antibody as a control were analyzed by a competitive assay. In brief, the unlabeled target antibody was allowed to interact with the TIM-3 expression cell prepared in Example 1, and whether or not other fluorescence labeled anti-TIM-3 antibody was further bound to TIM-3 expression cells were evaluated by flow cytometry method.

[0386] Human TIM-3 expressing EoL-1 cells were washed with staining medium and allowed to react with purified test monoclonal antibodies [antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 (manufactured by R&D Systems), antibody F38-2E2 (manufactured by Imgenex)] (4°C, 30 min). The final concentration was 10 μg/ml. Next, the Alexa-647 or APC labeled anti-TIM-3 antibodies [antibody 344823 (manufactured by R&D Systems) and antibody F38-2E2 (manufactured by eBiosciences)] were added and were allowed to react (4°C, 30 min). After washing with staining medium, 7-ADD (manufactured by BD Biosciences) was added before analyzing with FACSCalibur (man-

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ufactured by BD Biosciences).

[0387] The results are shown in Table 1. As shown in Table 1, the case the degree of the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was similar to that of a negative control were indicated as "+", the case where the degree to the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was found but was lower than that of a negative control was indicated as "+/-"and the case where the binding to the TIM-3 expression cell to which the unlabeled anti-TIM-3 antibody was bound was not detected, it was indicated as "-".

[0388]

[Table 1]

		Unlabeled antibody						
		DNP	512	644	4545	8213	344823	F38-2E2
Alexa-647 or APC labeled antibody	DNP	-	-	-	-	-	-	-
	512	+	-	+	+	+	+	+
	644	+	+	+/-	+	+	+/-	+/-
	4545	+	+	+	-	-	+	+
	8213	+	+	+	-	-	+	+
	344823	+	+	-	+	+	-	+/-
	F38-2E2	+	+	-	+	+	-	-

[0389] As shown in Table 1, since Alexa-647 labeled antibody 512 was found to bind to the TIM-3 expressing cell to which the other unlabeled anti-TIM-3 antibodies were bound, this antibody was suggested to recognize an independent epitope with which antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 did not compete.

[0390] Since it was found that Alexa-647 labeled antibody 644 did not bind or reduced in binding to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 344823 or F38-2E2 was bound, it was indicated that this antibody competed with antibody 344823 or F38-E2E in the recognition of the epitope close to each other.

[0391] Since it was found that Alexa-647 labeled antibody 4545 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 8213 was bound, it was indicated that this antibody competed with antibody 8213 in the recognition of the epitope close to each other.

[0392] Since it was found that Alexa-647 labeled antibody 8213 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 4545 was bound, it was indicated that this antibody competed with antibody 4545 in the recognition of the epitope close to each other.

[0393] Since it was found that APC labeled antibody 344823 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 644 or F38-2E2 was bound, it was indicated that this antibody competed with antibody 644 or F38-E2E antibody in the recognition of the epitope close to each other.

[0394] Since it was found that APC labeled Antibody F38-2E2 did not bind to the TIM-3 expressing cell to which the unlabeled anti-TIM-3 antibody 644 or 344823 was bound, it was indicated that this antibody competed with antibody 644 or antibody 344823 in the recognition of the epitope close to each other.

[Example 20]

(Molecular cloning of mouse TIM-3 cDNA)

[0395] The cDNA of mouse TIM-3 was prepared in the same manner as described in Example 1. Total RNA was extracted from Balb/c mouse derived bone marrow cells using High Pure RNA isolation Kit (manufactured by Roche). Template cDNA was synthesized using ThermoScript RT-PCR system (manufactured by Invitrogen). For primers, mTim-3 Fw3 (SEQ ID NO: 70) and mTim-3 Re3 (SEQ ID NO: 71) were used. The cDNA was inserted into pGEM-T Easy Vector (manufactured by Promega Corp.), and the sequence was analyzed. The clone having identical nucleotide sequence with the coding region of GenBank accession number AF450241 was selected.

[0396] PCR was carried out using the selected clone as the template and inserting the DNA into NotI site of the pEF6/Myc-HisC vector (manufactured by Invitrogen) (mTim-3/pEF6 Myc_HisC). For PCR primers, mTim-3 Fw4NotI (SEQ ID NO: 72) and mTim-3 Re4NotI (SEQ ID NO: 73) were used.

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[Example 21]

(Construction of hTim-3/pEF6 Myc_HisC)

[0397] The plasmid DNA hTIM-3/pMCs-IG prepared in Example 1 and pEF6 Myc_HisC were both digested with NotI to construct human TIM-3 expressing pEF6 Myc_HisC vector (hTim-3/pEF6 Myc_HisC). DNA sequence analysis confirmed that the vector sequence matched the coding region of the GenBank accession number NM_032782.

[Example 22]

(Cross-reactivity of anti-human TIM-3 antibody to anti-mouse TIM-3 antibody)

[0398] Each of the plasmid DNAs, mTim-3/pEF6 Myc_HisC and hTim-3/pEF6 Myc_HisC prepared in Example 20 and Example 21, and an empty vector control was introduced into HEK293F cell in the same manner as Example 4. Two days later, in the same manner as Example 15, the binding activity of Alexa-647 labeled antibodies (antibody 512, antibody 644, antibody 4545 and antibody 8213), commercially available APC labeled anti-human TIM-3 antibodies (antibody 344823 and antibody F38-2E2) and commercially available PE labeled anti-mouse TIM-3 antibody (RMT3-23 antibody, manufactured by BioLegend Inc.) to human TIM-3 and mouse TIM-3 was evaluated using flow cytometry analysis.

[0399] As a result, antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 exclusively interacted with human TIM-3 expressing 293F cells. However, RMT3-23 antibody interacted exclusively with mouse TIM-3 expressing 293F cells. Therefore, it was found that antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2 did not cross-react with mouse TIM-3.

[Example 23]

(Construction of expression system for TIM-3 chimeric protein with IgV domain substituted for mouse TIM-3)

[0400] The objective sequence was amplified by the PCR using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mIgV_vecR1 primer (SEQ ID NO: 74) and hTIM3+mIgV_vecF1 primer (SEQ ID NO: 75), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The objective sequence was amplified by PCR using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mIgV_insF1 primer (SEQ ID NO: 76) and hTIM3+mIgV_insR1 primer (SEQ ID NO: 77), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0401] The two PCR products obtained were ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence from the transformant was analyzed and it was found that the PCR products had the objective sequences (IgV chimeraTIM-3/pEF6 Myc_HisC, SEQ ID NO: 78).

[Example 24]

(Construction of expression system for TIM-3 chimeric protein with Mucin domain substituted for mouse TIM-3)

[0402] The objective sequence was amplified by PCR using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mMucin_vecR2 primer (SEQ ID NO: 80) and hTIM3+mMucin_vecF2 primer (SEQ ID NO: 81), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The objective sequence was amplified by PCR using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3+mMuicn_insF2 primer (SEQ ID NO: 82) and hTIM3+mMucin_insR2 primer (SEQ ID NO: 83), and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0403] The two PCR products obtained were ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence from the transformant was analyzed and it was found that the PCR products had the objective sequences(Mucin chimeraTIM-3/pEF6 Myc_HisC, SEQ ID NO: 84).

[Example 25]

(Binding assay of anti-TIM-3 antibody to TIM-3 chimeric protein in which IgV domain was substituted with mouse TIM-3 and TIM-3 chimeric protein in which Mucin domain was substituted with mouse TIM-3)

[0404] Each of the plasmid DNAs prepared in Example 23 and Example 24, IgV chimeraTIM-3/pEF6 Myc_HisC, Mucin chimeraTIM-3/pEF6 Myc_HisC, hTim-3/pEF6 Myc_HisC, mTim-3/pEF6 Myc_HisC, and an empty vector control was introduced into HEK293F cell in the same manner as Example 4. Two days later, in the same manner as Example 22,

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the binding activity of anti-TIM-3 antibody was evaluated using flow cytometry.

[0405] As a result, antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 4177, antibody 344823 and antibody F38-2E2 bound to the human TIM-3 expressing cells and Mucin chimeraTIM-3 expressing cells only. However, RMT-23 antibody bound to the mouse TIM-3 expressing cells and the IgV chimeraTIM-3 expressing cells only.

[0406] Therefore, as a result, it was found that all of the anti-human TIM-3 antibodies tested (antibody 512, antibody 644, antibody 4545, antibody 8213, antibody 344823 and antibody F38-2E2) and mouse TIM-3 antibody (antibody RMT3-23) recognized the IgV domain.

[Example 26]

(Construction of TIM-3 chimera 22-47/pEF6 Myc_HisC)

[0407] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 22 to position 47 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 22-47") was constructed. By using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, and hTIM3chimera22-47F1 primer (SEQ ID NO: 86) and hTIM3chimera22-47R1 primer (SEQ ID NO: 87), and PCR was carried out using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0408] Using the obtained PCR product as a template, the objective sequence was amplified by PCR using hTIM3chimera22-47F2 primer (SEQ ID NO: 88)and hTIM3chimera22-47R2 primer (SEQ ID NO: 89) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0409] Using the obtained PCR product as a template, the objective sequence was amplified by PCR using hTIM3chimera22-47F3 primer (SEQ ID NO: 90) and hTIM3chimera22-47R3 primer (SEQ ID NO: 91), and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0410] After the obtained PCR product obtained from the third PCR was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated using GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that the clone had the objective sequence (TIM-3 chimera 22-47/pEF6 Myc_HisC, SEQ ID NO: 92).

[Example 27]

(Construction of TIM-3 chimera 57-66/pEF6 Myc_HisC)

[0411] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 57 to position 66 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 57-66") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera57-66F primer (SEQ ID NO: 94) and hTIM3chimera57-66R primer (SEQ ID NO: 95) phosphorylated by T4 Polynucleotide Kinase (manufactured by New England Biolabs) and PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.) to amplify the objective sequence.

[0412] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to the agarose gel electrophoresis. The DNA was extracted and ligated with LigaFast™ Rapid DNA Ligation System (manufactured by Promega Corp.). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 57-66/pEF6 Myc_HisC, SEQ ID NO: 96).

[Example 28]

(Construction of TIM-3 chimera 67-105/pEF6 Myc_HisC)

[0413] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 67 to position 105 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 67-105") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA as a template, hTIM3chimera67-105F primer (SEQ ID NO: 98) and hTIM3chimera67-105R primer (SEQ ID NO: 99) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.) to amplify the objective sequence..

[0414] PCR was carried out using mTim-3/pEF6 Myc_HisC plasmid DNA as a template, mTIM3chimera67-105F primer (SEQ ID NO: 100) and mTIM3chimera67-105R primer (SEQ ID NO: 101) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.). The two PCR products were ligated by GENEART seamless cloning and assembly kit (manufactured by Invitorgen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 67-105/pEF6 Myc_HisC, SEQ ID NO: 102).

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[Example 29]

(Construction of TIM-3 chimera 74-81/pEF6 Myc_HisC)

[0415] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 74 to position 81 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 74-81") was constructed. PCR was carried out using hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3 chimera74-8 1 F primer (SEQ ID NO: 104) and hTIM3chimera74-81R primer (SEQ ID NO: 105) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0416] After the PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 74-81/pEF6 Myc_HisC, SEQ ID NO: 106).

[Example 30]

(Construction of TIM-3 chimera 88-96/pEF6 Myc_HisC)

[0417] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 88 to position 96 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as " TIM-3 chimera 88-96") was constructed. The PCR was carried out by preparing a reaction solution containing hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera88-96F primer (SEQ ID NO: 108) and hTIM3chimera88-96R primer (SEQ ID NO: 109) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0418] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 88 -96/pEF6 Myc_HisC, SEQ ID NO: 110).

[Example 31]

(Construction of TIM-3 chimera 96-105/pEF6 Myc_HisC)

[0419] A vector which expressed TIM-3 chimeric protein in which the amino acid of human TIM-3 (SEQ ID NO: 53) at position 96 to position 105 were substituted with the corresponding amino acid of mouse TIM-3 (hereinafter referred to as "TIM-3 chimera 96-105") was constructed. The PCR was carried out by preparing a reaction solution containing hTim-3/pEF6 Myc_HisC plasmid DNA prepared in Example 21 as a template, hTIM3chimera96-105F primer (SEQ ID NO: 112) and hTIM3chimera96-105R primer (SEQ ID NO: 113) and using PrimeSTAR GXL DNA Polymerase (manufactured by Takara Bio Inc.).

[0420] After the obtained PCR product was digested with *DpnI* (manufactured by New England Biolabs), it was subjected to agarose gel electrophoresis. The DNA was extracted and ligated with GENEART seamless cloning and assembly kit (manufactured by Invitrogen). The sequence of the clone obtained from the transformant was analyzed and confirmed that it was the objective sequence (TIM-3 chimera 96 - 105/pEF6 Myc_HisC, SEQ ID NO: 114).

[Example 32]

(Binding assay of anti-TIM-3 antibody to TIM-3 chimeric protein in which part of the IgV domain was substituted with mouse TIM-3)

[0421] Each of various TIM-3 vectors prepared in Examples 26 to 31 and an empty vector as a control were introduced into HEK293F in the same manner as Example 4. The introduced vectors and the expressed TIM-3 chimeric proteins thereof were as follows:

(1) TIM-3 chimera 22-47: TIM-3 chimeric protein in which the amino acid sequence at position 22 to position 47 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(2) TIM-3 chimera 57-66: TIM-3 chimeric protein in which the amino acid sequence at position 57 to position 66 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(3) TIM-3 chimera 67-105: TIM-3 chimeric protein in which the amino acid sequence at position 67 to position 105

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of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(4) TIM-3 chimera 74-81: TIM-3 chimeric protein in which the amino acid sequence at position 74 to position 81 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3;
(5) TIM-3 chimera 88-96: TIM-3 chimeric protein in which the amino acid sequence at position 88 to position 96 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3; and
(6) TIM-3 chimera 96-105: TIM-3 chimeric protein in which the amino acid sequence at position 96 to position 105 of human TIM-3 (SEQ ID NO: 53) were substituted with the corresponding amino acids of mouse TIM-3.

Two days after transfection, in the same manner as Example 22, the binding activity of anti-TIM-3 antibody was evaluated using flow cytometry.

[0422] The results are shown in Table 2. As shown in Table 2, the case where the binding of the antibody to the transfected cell was detected was indicated as "+", the case where the binding of the antibody to the transfected cell was detected but the degree of the binding was reduced was indicated as "+/-"and the case where the binding of the antibody to the trasfected cell was not detected was indicated as "-".

[Table 2]

Vector name	4545	8213	4177	512	644	344823	F38-2E2
TIM-3 chimera 22-47	+	+	+/-	-	+	+	+
TIM-3 chimera 57-66	+	+	+/-	+	-	-	-
TIM-3 chimera 67-105	-	-	+/-	+	+	+	+
TIM-3 chimera 74-81	-	-	+	+	+	+	+
TIM-3 chimera 88-96	+	+	+	+	+	+	+
TIM-3 chimera 96-105	+	+	+	+	+	+	+

[0423] As shown in Table 2, antibody 4545 and antibody 8213 bound to the cells transfected with TIM3-chimera 22-47, TIM3-chimera 57-66, TIM3-chimera 88-96 or TIM3-chimera 96-105 transfected cells. However, these antibodies did not bind to the cell into which TIM-3 chimera 67-105 were introduced.

[0424] The antibody 512 bound to the cells transfected with TIM3-chimera 57-66, TIM3-chimera 88-96, TIM3-chimera 96-105, TIM-3 chimera 67-105 or TIM-3 chimera 74-81. However, antibody 512 did not bind to the cells transfected with TIM-3 chimera 22-47.

[0425] The antibody 644, antibody 344823 and antibody F38-2E2 bound to the cells transfected with TIM3-chimera 22-47, TIM3-chimera 88-96, TIM3-chimera 96-105, TIM-3 chimera 67-105 or TIM-3 chimera 74-81. However, these antibodies did not bind to the cells transfected with to TIM-3 chimera 57-66.

The antibody 4177 bound to the cells transfected with TIM3-chimera 88-96, TIM3-chimera 96-105 or TIM-3 chimera 74-81. Antibody 4177 weakly bound to TIM-3 the cells transfected with chimera 22-47, TIM-3 chimera 57-66 or TIM-3 chimera 67-105.

[0426] Based on the above result and results from Example 16 and Example 19, it was suggested that antibody 4545 and antibody 8213 bound to the amino acid sequence at position 67 to 87 of human TIM-3. Since antibody 4545 and antibody 8213 did not bind to the cells transfected with TIM-3 chimera 74-81, it was suggested that the amino acids required for binding of antibody 4545 and antibody 8213 to human TIM-3 were included in the amino acid at position 74 to 81.

[0427] In addition, it was suggested that antibody 512 bound to the amino acids up to at position 47 of human TIM-3 and that antibody 644, antibody 344823 and antibody F38- 2E2 bound to the amino acid sequence at position 57 to 66 of human TIM-3.

[0428] Furthermore, it was suggested that antibody 4177 bound to the amino acids other than at position 74 to 81 within the amino acids at position 67 to 87 of human TIM-3 to which antibody 4545 and antibody 8213 bind although antibody 512, antibody 644, antibody 344823 and antibody F38-2E2 did not bind.

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[Example 33]

(Preparation of anti-human TIM-3 antibody 8213 humanized antibody)

(1) Design of amino acid sequences of VH and VL of antibody 8213 humanized antibody

[0429] The amino acid sequence of VH of antibody 8213 humanized antibody was designed in the following manner. The amino acid sequences of FRs in VH of human antibody which is suitable for the grafting of the amino acid sequences (SEQ ID NOs: 21 to 23) of CDRs1 to 3 of antibody 8213 were selected as follows.

[0430] Kabat *et al.,* have classified the VH of conventionally known various human antibodies into three subgroups (HSG I to III) based on the homology of their amino acid sequences and reported the consensus sequences for each of the subgroups [*Sequences of Proteins of Immunological Interest,* US Dept. Health and Human Services (1991)]. Therefore, the homology search of the amino acid consensus sequences of FR of VH subgroups I to III of human antibodies with the amino sequence of FR ofVH of antibody 8213 was conducted.

[0431] As a result of the homology search, the homologies of HSGI, HSGII, and HSGIII were 77.0%, 55.2%, and 58.6%, respectively. Therefore, the amino acid sequence of FR of the VH region of antibody 8213 had the highest homology to the subgroup I.

[0432] Based on the above results, the amino acid sequence of CDR of the VH of antibody 8213 (SEQ ID NOs: 21 to 23) was grafted to an appropriate position of the FR amino acid sequence of the consensus sequence of subgroup I of human antibody VH. In this manner, the 8213 antibody HV0 represented by SEQ ID NO: 67, i.e., the amino acid sequence of VH of an anti-human TIM-3 antibody 8213 humanized antibody was designed.

[0433] Next, the amino acid sequence of VL of antibody 8213 humanized antibody was designed in the following manner. The amino acid sequences of FR in VL of human antibody which were suitable for the grafting of the amino acid sequences (SEQ ID NOs: 24 to 26) of CDRs1 to 3 in VL of antibody 8213 were selected as follows.

[0434] Kabat *et al.,* have classified the VL of various known human antibodies into four subgroups (HSG I to IV) based on the homology of their amino acid sequences and reported the consensus sequences for each of the subgroups [Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)]. Therefore, the homology search among the FR amino acid consensus sequences of VL subgroup I to IV of human antibodies were searched for sequence homology with FR amino sequence of VL of antibody 8213.

[0435] As a result of the homology search, the homologies of HSGI, HSGII, HSGIII and HSGIV were 76.3%, 61.3%, 61.3%, and 68.8%, respectively. Therefore, the amino acid sequence of FR of the VL region of antibody 8213 had the highest homology to the subgroup I.

[0436] Based on the above results, each of the amino acid sequences of CDRs of the VL of antibody 8213 (SEQ ID NOs: 24 to 26) was grafted into an appropriate position in the amino acid sequence of FR of the consensus sequence of subgroup I of VL of an human antibody. In this manner, the 8213 antibody LV0 represented by SEQ ID NO: 69, i.e., the amino acid sequence of VL of an anti-human TIM-3 antibody 8213 humanized antibody, was designed.

[0437] The amino acid sequence of 8213 antibody HV0 which was VH of antibody 8213 humanized antibody; and the amino acid sequence of 8213 antibody LV0 which was VL of antibody 8213 humanized antibody designed in the above were the sequences in which only the amino acid sequences of CDRs of the mouse monoclonal antibody 8213 were grafted into the amino acid sequence of the selected FR of human antibody.

[0438] However, in general, it is known that a humanized antibody prepared merely by grafting CDRs of a mouse antibody to FRs of a human antibody has a lower binding activity. In order to avoid decreasing of the binding activity, attempts have been made in the preparation of a humanized antibody to raise the lowered binding activity by modifying the amino acid residues which were considered to have influence on the binding activity among the amino acid sequences of FRs of a human antibody which are different from a mouse antibody as well as grafting amino acid sequences of CDRs. Therefore, in Examples, the inventors decided to identify and modify the amino acid residues of FR which were considered to have influence on the binding activity in the following manner.

[0439] First, three-dimensional structure of the above designed antibody V region (hereinafter referred to as " HV0LV0") comprising the amino acid sequence of antibody 8213 HV0 which was VH of the antibody 8213 humanized antibody and the amino acid sequence of antibody 8213 LV0 which was VL of antibody 8213 humanized antibody was constructed using a computer modeling technique. Discovery Studio (manufactured by Accelrys Inc.) was used for preparation of the three-dimensional structure and display of the three-dimensional structure. A computer model of the three-dimensional structure of V region of antibody 8213 was constructed in the same manner.

[0440] Furthermore, amino acid residues which were different from those of antibody 8213 in the amino acid sequence of FRs ofVH and VL of HV0LV0 were selected, prepared an amino acid sequence in which such amino acid residues were substituted with the corresponding amino acid residues of 8213 antibody and then a three-dimensional structure model was constructed in the same manner. The amino acids which were considered to have influence on the binding activity were selected by comparing the three-dimensional structures of the V regions of antibody 8213 and HV0LV0

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and the modified product.

[0441] As a result, as amino acid residues of FR in HV0LV0 which were considered to change the three-dimensional structure of the antigen binding region and have influence on the binding activity, Lys at position 12, Val at position 20, Arg at position 38, Ala at position 40, Met at position 48, Arg at position 67, Val at position 68, Ile at position 70, Ala at position 72, Thr at position 74, Arg at position 98 and Val at position 113 were selected in antibody 8213 HV0; and Leu at position 11, Ala at position 13, Val at position 15, Tyr at position 36, Ala at position 43, Pro at position 44, Leu at position 46, Phe at position 71 and Thr at position 85 were selected in antibody 8213 LV0.

[0442] Among the selected amino acid residues, at least one or more amino acid residues was substituted with amino acid residues which were present at the corresponding sites of antibody 8213 and VHs and VLs of the humanized antibody comprising various modifications were constructed.

[0443] Specifically, as for VH, at least one modification was introduced among the amino acid modifications for substituting Lys at position 12 with Val, Val at position 20 with Leu, Arg at position 38 with Lys, Ala at position 40 with Arg, Met at position 48 with Ile, Arg at position 67 with Lys, Val at position 68 with Ala, Ile at position 70 with Leu, Ala at position 72 with Val, Thr at position 74 with Lys, Arg at position 98 with Gly, or Val at position 113 with Leu in the amino acid sequence represented by SEQ ID NO: 67.

[0444] As for VL, at least one modification was introduced among the amino acid modifications for substituting Leu at position 11 with Met, Ala at position 13 with Val, Val at position 15 with Leu, Tyr at position 36 with Leu, Ala at position 43with Ser, Pro at position 44 with Phe, Leu at position 46 with Gly, Phe at position 71 with Tyr and Thr at position 85 with Asp in the amino acid sequence represented by SEQ ID NO: 69.

[0445] As the antibody V region of 8213 antibody-humanized antibody in which at least one amino acid existing in FR in HV0LV0 was modified, HV0LV0, HV0LV2, HV0LV4, HV0LV5, HV0LV6, HV0LV7, HV0LV9, HV3LV0, HV3LV2, HV3LV4, HV3LV5, HV3LV6, HV3LV7, HV3LV9, HV4LV0, HV4LV2, HV4LV4, HV4LV5, HV4LV6, HV4LV7, HV4LV9, HV5LV0, HV5LV2, HV5LV4, HV5LV5, HV5LV6, HV5LV7, HV5LV9, HV6LV0, HV6LV2, HV6LV4, HV6LV5, HV6LV6, HV6LV7, HV6LV9, HV7LV0, HV7LV2, HV7LV4, HV7LV5, HV7LV6, HV7LV7, HV7LV9, HV8LV0, HV8LV2, HV8LV4, HV8LV5, HV8LV6, HV8LV7, HV8LV9, HV10LV0, HV10LV2, HV10LV4, HV10LV5, HV10LV6, HV10LV7, HV10LV9, HV12LV0, HV12LV2, HV12LV4, HV12LV5, HV12LV6, HV12LV7, and HV12LV9 were designed.

[0446] The amino acid sequences of each of the H chain variable regions, HV3, HV4, HV5, HV6, HV7, HV8, HV10 and HV12; and the amino acid sequence of the L chain variable region, LV2, LV4, LV5, LV6, LV7 and LV9 were shown in Fig. 1 and Fig. 2, respectively.

(2) Preparation of 8213 antibody-humanized antibody

[0447] DNA encoding the amino acid sequence of the variable region of 8213 antibody-humanized antibody was designed using codons used in the DNAs (SEQ ID NO: 27 and 29) encoding the amino acid sequence of VH of 8213 antibody and VL of 8213 antibody, respectively. When the amino acid modification was introduced, DNA was designed using a codon which was used in a mammal cell with a high frequency.

[0448] Using these DNA sequences, vectors for expressing an antibody were constructed and humanized antibodies were expressed.

[0449] While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

This application is based on U.S. provisional application No. 61/353,836, filed on June 11, 2010, the entire contents of which are incorporated hereinto by reference. All references cited herein are incorporated in their entirety.

Industrial Applicability

[0450] The persent invention can provide a monoclonal antibody or an antibody fragment thereof which binds to the amino acid sequence or the three-dimensional structure thereof of the extracellular region of human TIM-3 and has ADCC activity; a hybridoma which produces the antibody; a DNA which encods the antibody; a vector which comprises the DNA; a transformant which is obtained by transofrming the vector; a process for producing an antibody or an antibody fragment thereof using the hybridoma or the transformant; and a diagnostic agent or a therapeutic agent using the antibody or the antibody fragment thereof as an active ingredient.

Free text of Sequence Listing

[0451]

SEQ ID NO: 1: The amino acid sequence of Human 4545 H chain CDR1

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SEQ ID NO: 2: The amino acid sequence of Human 4545 H chain CDR2
SEQ ID NO: 3: The amino acid sequence of Human 4545 H chain CDR3
SEQ ID NO: 4: The amino acid sequence of Human 4545 L chain CDR1
SEQ ID NO: 5: The amino acid sequence of Human 4545 L chain CDR2
SEQ ID NO: 6: The amino acid sequence of Human 4545 L chain CDR3
SEQ ID NO: 11: The amino acid sequence of Human 4177 H chain CDR1
SEQ ID NO: 12: The amino acid sequence of Human 4177 H chain CDR2
SEQ ID NO: 13: The amino acid sequence of Human 4177 H chain CDR3
SEQ ID NO: 14: The amino acid sequence of Human 4177 L chain CDR1
SEQ ID NO: 15: The amino acid sequence of Human 4177 L chain CDR2
SEQ ID NO: 16: The amino acid sequence of Human 4177 L chain CDR3
SEQ ID NO: 21: The amino acid sequence of Mouse 8213 H chain CDR1
SEQ ID NO: 22: The amino acid sequence of Mouse 8213 H chain CDR2
SEQ ID NO: 23: The amino acid sequence of Mouse 8213 H chain CDR3
SEQ ID NO: 24: The amino acid sequence of Mouse 8213 L chain CDR1
SEQ ID NO: 25: The amino acid sequence of Mouse 8213 L chain CDR2
SEQ ID NO: 26: The amino acid sequence of Mouse 8213 L chain CDR3
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SEQ ID NO: 32: The nucleotide sequence of primer TIM-3 Re2
SEQ ID NO: 33: The nucleotide sequence of primer pMCs-Fw
SEQ ID NO: 34: The nucleotide sequence of primer TIM3ED-FcReXba
SEQ ID NO: 35: The nucleotide sequence of primer T7
SEQ ID NO: 36: The nucleotide sequence of primer hTIM-3 Fw1
SEQ ID NO: 37: The nucleotide sequence of insert hTIM-3 ECD Fc fusion
SEQ ID NO: 38: The amino acid sequence of insert hTIM-3 ECD Fc fusion
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SEQ ID NO: 43: The amino acid sequence of insert hTIM-3 ECD
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SEQ ID NO: 45: The nucleotide sequence of primer hh-3
SEQ ID NO: 46: The nucleotide sequence of primer hk-2
SEQ ID NO: 47: The nucleotide sequence of primer hk-6
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SEQ ID NO: 67: The amino acid sequence of 8213 antibody HV0 variable region
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SEQ ID NO: 73: The nucleotide sequence of primer mTim-3 Re4NotI
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SEQ ID NO: 111: The amino acid sequence of insert TIM-3 chimera 88-96/pEF6 Myc_HisC
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SEQ ID NO: 114: The nucleotide sequence of insert TIM-3 chimera 96-105/pEF6 Myc_HisC
SEQ ID NO: 115: The amino acid sequence of insert TIM-3 chimera 96-105/pEF6 Myc_HisC

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Kyushu University, National University Corporation

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atg gac atg agg gtc ctc gct cag ctc ctg ggg ctc ctg ctg ctc tgt       48
Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15

ttc cca ggt gcc aga tgt gac atc cag atg acc cag tct cca tcc tca       96
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgt cgg gcg agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggt att agc agc tgg tta gcc tgg tat cag cag aaa cca gag aaa      192
Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

gcc cct aag tcc ctg atc tat gct gca tcc agt ttg caa agt ggg gtc      240
Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgc caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

tat aat agt tac cct cgg acg ttc ggc caa ggg acc aag gtg gaa atc      384
Tyr Asn Ser Tyr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
        115                 120                 125

aaa                                                                  387
Lys


<210>  20
<211>  129
<212>  PRT
<213>  Homo sapiens

<400>  20
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```
Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                  10                  15

Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

Tyr Asn Ser Tyr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
        115                 120                 125

Lys
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<210> 21
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<220>
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<400> 21

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Ser Tyr Trp Met His
1               5
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<210> 22
<211> 17
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 H-chain CDR2

<400> 22

```
Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe Lys
1               5                  10                  15
```


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Thr

<210> 23
<211> 8
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 H-chain CDR3

<400> 23

Gly Tyr Tyr Leu Tyr Phe Asp Tyr
1 5

<210> 24
<211> 11
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR1

<400> 24

His Ala Ser Gln Gly Ile Arg Ile Asn Ile Gly
1 5 10

<210> 25
<211> 7
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR2

<400> 25

His Gly Thr Asn Leu Glu Asp
1 5

<210> 26
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Mouse8213 L-chain CDR3

<400> 26

Val Gln Tyr Gly Gln Phe Pro Trp Thr
1 5

<210> 27
<211> 408

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<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(408)

<400> 27

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atg gga tgg agc tat atc atc ctc ttt ttg gta gca aca gcg aca gat       48
Met Gly Trp Ser Tyr Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Asp
1               5                   10                  15

gtc cac tcc cag gtc caa ctg cag cag cct ggg gct gaa ctg gtg aag       96
Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys
            20                  25                  30

cct ggg gct tca gtg aag ctg tcc tgc aag gct tct ggc tac acc ttc      144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

acc agc tac tgg atg cac tgg gtg aag cag agg cct gga caa ggc ctt      192
Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg att gga gag att aat cct agc aac ggt cgt act aac tac aat      240
Glu Trp Ile Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
65                  70                  75                  80

gag aag ttc aag acc aag gcc aca ctg act gta gac aaa tcc tcc agc      288
Glu Lys Phe Lys Thr Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
                85                  90                  95

aca gcc tac atg caa ctc agc agc ctg aca tct gag gac tct gcg gtc      336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
            100                 105                 110

tat tac tgt gcg ggg ggt tac tac ctc tac ttt gac tac tgg ggc caa      384
Tyr Tyr Cys Ala Gly Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln
        115                 120                 125

ggc acc act ctc aca gtc tcc tca                                     408
Gly Thr Thr Leu Thr Val Ser Ser
    130                 135
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<210> 28
<211> 136
<212> PRT
<213> Mus musculus

<400> 28

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Met Gly Trp Ser Tyr Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Asp
1               5                   10                  15

Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45
```


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Thr Ser Tyr Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
65 70 75 80

Glu Lys Phe Lys Thr Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Tyr Cys Ala Gly Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln
115 120 125

Gly Thr Thr Leu Thr Val Ser Ser
130 135

<210> 29
<211> 387
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(387)

<400> 29

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Met Asp Met Met Val Leu Ala Gln Phe Leu Ala Phe Leu Leu Leu Trp
1               5                   10                  15

ttt cca ggt gca aca tgt gac atc ctg atg acc caa tct cca tcc tcc       96
Phe Pro Gly Ala Thr Cys Asp Ile Leu Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

atg tct gta tct ctg gga gac aca gtc aac atc act tgc cat gca agt      144
Met Ser Val Ser Leu Gly Asp Thr Val Asn Ile Thr Cys His Ala Ser
        35                  40                  45

cag ggc att agg att aat ata ggg tgg ttg cag cag aag cca ggg aaa      192
Gln Gly Ile Arg Ile Asn Ile Gly Trp Leu Gln Gln Lys Pro Gly Lys
    50                  55                  60

tca ttt aag ggc ctg atc tat cat gga acc aac ttg gaa gat gga gtt      240
Ser Phe Lys Gly Leu Ile Tyr His Gly Thr Asn Leu Glu Asp Gly Val
65                  70                  75                  80

cca tca agg ttc agt ggc agt gga tct gga cca gat tat tct ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Pro Asp Tyr Ser Leu Thr
                85                  90                  95

atc agc agc ctg gaa tct gaa gat ttt gca gac tat tac tgt gta cag      336
Ile Ser Ser Leu Glu Ser Glu Asp Phe Ala Asp Tyr Tyr Cys Val Gln
```


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100 105 110

tat ggt cag ttt ccg tgg acg ttc ggt gga ggc acc aag ctg gaa atg 384
Tyr Gly Gln Phe Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Met
115 120 125

aaa 387
Lys

<210> 30
<211> 129
<212> PRT
<213> Mus musculus

<400> 30

Met Asp Met Met Val Leu Ala Gln Phe Leu Ala Phe Leu Leu Leu Trp
1 5 10 15

Phe Pro Gly Ala Thr Cys Asp Ile Leu Met Thr Gln Ser Pro Ser Ser
20 25 30

Met Ser Val Ser Leu Gly Asp Thr Val Asn Ile Thr Cys His Ala Ser
35 40 45

Gln Gly Ile Arg Ile Asn Ile Gly Trp Leu Gln Gln Lys Pro Gly Lys
50 55 60

Ser Phe Lys Gly Leu Ile Tyr His Gly Thr Asn Leu Glu Asp Gly Val
65 70 75 80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Pro Asp Tyr Ser Leu Thr
85 90 95

Ile Ser Ser Leu Glu Ser Glu Asp Phe Ala Asp Tyr Tyr Cys Val Gln
100 105 110

Tyr Gly Gln Phe Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Met
115 120 125

Lys

<210> 31
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer TIM-3 Fw2

<400> 31

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gccaccatgt tttcacatct tccctt 26

<210> 32
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer TIM-3 Re2

<400> 32
ctatggcatt gcaaagcgac 20

<210> 33
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Primer pMCs-Fw

<400> 33
tcaaagtaga cggcatcgca g 21

<210> 34
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer TIM3ED-FcReXba

<400> 34
ttttctagat ctgatggttg ctccaga 27

<210> 35
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer T7

<400> 35
taatacgact cactataggg 20

<210> 36
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM-3 Fw1

<400> 36
actctggagc aaccatca 18

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<210> 37
<211> 1333
<212> DNA
<213> Artificial

<220>
<223> Insert hTIM-3 ECD Fc fusion

<220>
<221> CDS
<222> (11)..(1333)

<400> 37
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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu
1 5 10

ctg ctg cta cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag 97
Leu Leu Leu Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu
15 20 25

gtc ggt cag aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca 145
Val Gly Gln Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro
30 35 40 45

ggg aac ctc gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt 193
Gly Asn Leu Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe
50 55 60

gaa tgt ggc aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat 241
Glu Cys Gly Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr
65 70 75

tgg aca tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg 289
Trp Thr Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val
80 85 90

tcc ctg acc ata gag aat gtg act cta gca gac agt ggg atc tac tgc 337
Ser Leu Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys
95 100 105

tgc cgg atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg 385
Cys Arg Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu
110 115 120 125

aag ttg gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag 433
Lys Leu Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln
130 135 140

aga gac ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat 481
Arg Asp Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His
145 150 155

ggc cca gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta 529
Gly Pro Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu
160 165 170

aca caa ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc 577
Thr Gln Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala
175 180 185

aat gac tta cgg gac tct gga gca acc atc aga tct aga gca gac tac 625

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```
Asn Asp Leu Arg Asp Ser Gly Ala Thr Ile Arg Ser Arg Ala Asp Tyr
190                 195                 200                 205

aag gac gac gat gac aag act agt gac aaa act cac aca tgc cca ccg      673
Lys Asp Asp Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro
                210                 215                 220

tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc      721
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
            225                 230                 235

cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca      769
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
        240                 245                 250

tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac      817
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
    255                 260                 265

tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg      865
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
270                 275                 280                 285

gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc      913
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
                290                 295                 300

ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc      961
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
            305                 310                 315

aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa     1009
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
        320                 325                 330

ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gag     1057
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu
    335                 340                 345

gag atg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc     1105
Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
350                 355                 360                 365

tat ccc agc gac atc gcc gcg gag tgg gag agc aat ggg cag ccg gag     1153
Tyr Pro Ser Asp Ile Ala Ala Glu Trp Glu Ser Asn Gly Gln Pro Glu
                370                 375                 380

aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc     1201
Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
            385                 390                 395

ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg     1249
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
        400                 405                 410

aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac     1297
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
    415                 420                 425

acg cag aag agc ctc tcc ctg tct ccg ggt aaa tga                    1333
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
430                 435                 440
```


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<210> 38
<211> 440
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 38

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ser Arg Ala Asp Tyr Lys Asp Asp
        195                 200                 205
```


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```
Asp Asp Lys Thr Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
    210                 215                 220

Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
225                 230                 235                 240

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                245                 250                 255

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
            260                 265                 270

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
        275                 280                 285

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
    290                 295                 300

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
305                 310                 315                 320

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                325                 330                 335

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr
            340                 345                 350

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
        355                 360                 365

Asp Ile Ala Ala Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
    370                 375                 380

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
385                 390                 395                 400

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
                405                 410                 415

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
            420                 425                 430

Ser Leu Ser Leu Ser Pro Gly Lys
        435                 440
```

<210> 39
<211> 30
<212> DNA

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<213> Artificial

<220>
<223> Primer TIM3ED-FLAG4aa

<400> 39
gtccttgtag tctctgatgg ttgctccaga 30

<210> 40
<211> 39
<212> DNA
<213> Artificial

<220>
<223> Primer C-FLAG-NotR2

<400> 40
aaaagcggcc gctcacttgt cgtcatcgtc cttgtagtc 39

<210> 41
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Primer BGH-R

<400> 41
tagaaggcac agtcgagg 18

<210> 42
<211> 645
<212> DNA
<213> Artificial

<220>
<223> Insert hTIM-3 ECD

<220>
<221> CDS
<222> (19)..(645)

<400> 42
gggaattcga ttgccacc atg ttt tca cat ctt ccc ttt gac tgt gtc ctg 51
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu
1 5 10

ctg ctg ctg ctg cta cta ctt aca agg tcc tca gaa gtg gaa tac aga 99
Leu Leu Leu Leu Leu Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg
15 20 25

gcg gag gtc ggt cag aat gcc tat ctg ccc tgc ttc tac acc cca gcc 147
Ala Glu Val Gly Gln Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala
30 35 40

gcc cca ggg aac ctc gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct 195
Ala Pro Gly Asn Leu Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro
45 50 55

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```
gtg ttt gaa tgt ggc aac gtg gtg ctc agg act gat gaa agg gat gtg      243
Val Phe Glu Cys Gly Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val
60                  65                  70                  75

aat tat tgg aca tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga      291
Asn Tyr Trp Thr Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly
                80                  85                  90

gat gtg tcc ctg acc ata gag aat gtg act cta gca gac agt ggg atc      339
Asp Val Ser Leu Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile
            95                  100                 105

tac tgc tgc cgg atc caa atc cca ggc ata atg aat gat gaa aaa ttt      387
Tyr Cys Cys Arg Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe
        110                 115                 120

aac ctg aag ttg gtc atc aaa cca gcc aag gtc acc cct gca ccg act      435
Asn Leu Lys Leu Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr
    125                 130                 135

cgg cag aga gac ttc act gca gcc ttt cca agg atg ctt acc acc agg      483
Arg Gln Arg Asp Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg
140                 145                 150                 155

gga cat ggc cca gca gag aca cag aca ctg ggg agc ctc cct gat ata      531
Gly His Gly Pro Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile
                160                 165                 170

aat cta aca caa ata tcc aca ttg gcc aat gag tta cgg gac tct aga      579
Asn Leu Thr Gln Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg
            175                 180                 185

ttg gcc aat gac tta cgg gac tct gga gca acc atc aga gac tac aag      627
Leu Ala Asn Asp Leu Arg Asp Ser Gly Ala Thr Ile Arg Asp Tyr Lys
        190                 195                 200

gac gat gac gac aag tga                                             645
Asp Asp Asp Asp Lys
    205
```

<210> 43
<211> 208
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 43

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45
```


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Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
165 170 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
180 185 190

Arg Asp Ser Gly Ala Thr Ile Arg Asp Tyr Lys Asp Asp Asp Asp Lys
195 200 205

<210> 44
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer hh-6

<400> 44
ggtccgggag atcatgaggg tgtcctt 27

<210> 45
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hh-3

<400> 45
gtgcacgccg ctggtcaggg cgcctg 26

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<210> 46
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hk-2

<400> 46
gttgaagctc tttgtgacgg gcgagc 26

<210> 47
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer hk-6

<400> 47
tggcgggaag atgaagacag atggtg 26

<210> 48
<211> 32
<212> DNA
<213> Artificial

<220>
<223> Primer mH_Rv1

<400> 48
attttgtcga cckyggtsyt gctggcyggg tg 32

<210> 49
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mH_Rv2

<400> 49
gcacacyrct ggacagggat ccagagttcc 30

<210> 50
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mK_Rv1

<400> 50
ttgaagctct tgacaatggg tgaagttgat 30

<210> 51
<211> 30

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<212> DNA
<213> Artificial

<220>
<223> Primer mK_Rv2

<400> 51
gtaggtgctg tctttgctgt cctgatcagt 30

<210> 52
<211> 906
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(906)

<400> 52
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc 384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc 432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca 480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata 528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile

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```
                  165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
```

<210> 53
<211> 301
<212> PRT
<213> Homo sapiens

<400> 53

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
```


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85 90 95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
130 135 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145 150 155 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
165 170 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
180 185 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
195 200 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
210 215 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225 230 235 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
245 250 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
260 265 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
275 280 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 54
<211> 426
<212> DNA
<213> Homo sapiens

<220>
<221> CDS

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<222> (1)..(426)

<400> 54

```
atg gag ttt ggg ctg agc tgg gtt ttc ctc gtt gct ctt tta aga ggt       48
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
1               5                   10                  15

gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc gtg gtc cag       96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
            20                  25                  30

cct ggg agg tcc ctg aga ctc tcc tgt gca gcg tct gga ttc acc ttc      144
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

aat agc tat ggc atg cac tgg gtc cgc cag gct cca ggc aag ggg ctg      192
Asn Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

gag tgg gtg gca gtt ata tgg tat gat gga agt aat aaa tac tat gga      240
Glu Trp Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Gly
65                  70                  75                  80

gac tcc gtg aag ggc cga ttc acc atc tcc aga gac aat tcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
                85                  90                  95

acg ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gct gtg      336
Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg ata tgg ttc ggg gag atg ttt tcc gaa tac ttc cag      384
Tyr Tyr Cys Ala Ile Trp Phe Gly Glu Met Phe Ser Glu Tyr Phe Gln
        115                 120                 125

cac tgg ggc cag ggc acc ctg gtc acc gtc tcc tca gct agc              426
His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 55
<211> 142
<212> PRT
<213> Homo sapiens

<400> 55

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Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
1               5                   10                  15

Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
            20                  25                  30

Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

Asn Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60
```


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```
Glu Trp Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Gly
65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
                85                  90                  95

Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Ile Trp Phe Gly Glu Met Phe Ser Glu Tyr Phe Gln
        115                 120                 125

His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 56
<211> 393
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(393)

<400> 56

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atg gac atg agg gtc ccc gct cag ctc ctg ggg ctt ctg ctg ctc tgg       48
Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp
1               5                   10                  15

ctc cca ggt gcc aga tgt gcc atc cag ttg acc cag tct cca tcc tcc       96
Leu Pro Gly Ala Arg Cys Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgc cgg gca agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggc att agc agt gct tta gcc tgg tat cag cag aaa cca ggg aaa      192
Gln Gly Ile Ser Ser Ala Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

gct cct aag ctc ctg atc tat gat gcc tcc agt ttg gaa agt ggg gtc      240
Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgt caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

ttt aat agt tac cct ctc act ttc ggc gga ggg acc aag gtg gag atc      384
Phe Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
        115                 120                 125
```


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aaa cgt acg 393
Lys Arg Thr
130

<210> 57
<211> 131
<212> PRT
<213> Homo sapiens

<400> 57

Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp
1 5 10 15

Leu Pro Gly Ala Arg Cys Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser
20 25 30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
35 40 45

Gln Gly Ile Ser Ser Ala Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys
50 55 60

Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val
65 70 75 80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
85 90 95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
100 105 110

Phe Asn Ser Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile
115 120 125

Lys Arg Thr
130

<210> 58
<211> 432
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(432)

<400> 58
atg gag ttt ggg ctg agc tgg gtt ttc ctt gtt gct att ata aaa ggt 48
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Ile Lys Gly
1 5 10 15

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```
gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc ttg gtc aag       96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys
            20                  25                  30

cct gga ggg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc      144
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

agt gac tac tac atg agc tgg atc cgc cag gct cca ggg aag ggg ctg      192
Ser Asp Tyr Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

gag tgg gtt tca ttc att agt agt agt ggt agt atc ata tac tac gca      240
Glu Trp Val Ser Phe Ile Ser Ser Ser Gly Ser Ile Ile Tyr Tyr Ala
65                  70                  75                  80

gac tct gtg aag ggc cga ttc acc atc tcc agg gac aac gcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95

tca ctg tat ctg caa atg aac agc ctg aga gcc gag gac acg gct gtg      336
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg aga gat ggg tat agc agc agt tgg tat tac tac ggt      384
Tyr Tyr Cys Ala Arg Asp Gly Tyr Ser Ser Ser Trp Tyr Tyr Tyr Gly
        115                 120                 125

atg gac gtc tgg ggc caa ggg acc acg gtc acc gtc tcc tca gct agc      432
Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 59
<211> 144
<212> PRT
<213> Homo sapiens

<400> 59

```
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Ile Lys Gly
1               5                   10                  15

Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys
            20                  25                  30

Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
        35                  40                  45

Ser Asp Tyr Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
    50                  55                  60

Glu Trp Val Ser Phe Ile Ser Ser Ser Gly Ser Ile Ile Tyr Tyr Ala
65                  70                  75                  80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
                85                  90                  95
```


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```
Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Asp Gly Tyr Ser Ser Ser Trp Tyr Tyr Tyr Gly
        115                 120                 125

Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser
    130                 135                 140
```

<210> 60
<211> 390
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(390)

<400> 60

```
atg gaa acc cca gcg cag ctt ctc ttc ctc ctg cta ctc tgg ctc cca       48
Met Glu Thr Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1               5                   10                  15

gat acc acc gga gaa att gtg ttg acg cag tct cca ggc acc ctg tct       96
Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser
            20                  25                  30

ttg tct cca ggg gaa aga gcc acc ctc tcc tgc agg gcc agt cag agt      144
Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
        35                  40                  45

gtt agc agc agc tac tta gcc tgg tac cag cag aaa cct ggc cag gct      192
Val Ser Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala
    50                  55                  60

ccc agg ctc ctc atc tat ggt gca tcc agc agg gcc act ggc atc cca      240
Pro Arg Leu Leu Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Ile Pro
65                  70                  75                  80

gac agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc      288
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                85                  90                  95

agc aga ctg gag cct gaa gat ttt gca gtg tat tac tgt cag cag tat      336
Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr
            100                 105                 110

ggt agc tca ccg ctc act ttc ggc gga ggg acc aag gtg gag atc aaa      384
Gly Ser Ser Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        115                 120                 125

cgt acg                                                             390
Arg Thr
    130
```

<210> 61
<211> 130

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<212> PRT
<213> Homo sapiens

<400> 61

Met Glu Thr Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro
1 5 10 15

Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser
20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser
35 40 45

Val Ser Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala
50 55 60

Pro Arg Leu Leu Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Ile Pro
65 70 75 80

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
85 90 95

Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr
100 105 110

Gly Ser Ser Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
115 120 125

Arg Thr
130

<210> 62
<211> 414
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(414)

<400> 62
atg gac tgg acc tgg agc atc ctt ttc ttg gtg gca gca gca aca ggt 48
Met Asp Trp Thr Trp Ser Ile Leu Phe Leu Val Ala Ala Ala Thr Gly
1 5 10 15

gcc cac tcc cag gtt cag ctg gtg cag tct gga gct gag gtg aag aag 96
Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

cct ggg gcc tca gtg aag gtc tcc tgc aag gct tct ggt tac acc ttt 144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

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```
acc agc tat ggt atc agc tgg gtg cga cag gcc cct gga caa ggg ctt      192
Thr Ser Tyr Gly Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

gag tgg atg gga tgg atc agc gct tac aat ggt aac aca aac tat gca      240
Glu Trp Met Gly Trp Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala
65                  70                  75                  80

cag aag ctc cag ggc aga gtc acc atg acc aca gac aca tcc acg agc      288
Gln Lys Leu Gln Gly Arg Val Thr Met Thr Thr Asp Thr Ser Thr Ser
                85                  90                  95

aca gcc tac atg gag ctg agg agc ctg aga tct gac gac acg gcc gtg      336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

tat tac tgt gcg aga tgg gat agc agc agc tgg tcc ttt gac tac tgg      384
Tyr Tyr Cys Ala Arg Trp Asp Ser Ser Ser Trp Ser Phe Asp Tyr Trp
        115                 120                 125

ggc cag gga acc ctg gtc acc gtc tcc tca                              414
Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135
```

<210> 63
<211> 138
<212> PRT
<213> Homo sapiens

<400> 63

```
Met Asp Trp Thr Trp Ser Ile Leu Phe Leu Val Ala Ala Ala Thr Gly
1               5                   10                  15

Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Ser Tyr Gly Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Met Gly Trp Ile Ser Ala Tyr Asn Gly Asn Thr Asn Tyr Ala
65                  70                  75                  80

Gln Lys Leu Gln Gly Arg Val Thr Met Thr Thr Asp Thr Ser Thr Ser
                85                  90                  95

Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Trp Asp Ser Ser Ser Trp Ser Phe Asp Tyr Trp
        115                 120                 125
```


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```
Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135


<210>  64
<211>  390
<212>  DNA
<213>  Homo sapiens


<220>
<221>  CDS
<222>  (1)..(390)

<400>  64
atg gac atg agg gtc ctc gct cag ctc ctg ggg ctc ctg ctg ctc tgt       48
Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15

ttc cca ggt gcc aga tgt gac atc cag atg acc cag tct cca tcc tca       96
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

ctg tct gca tct gta gga gac aga gtc acc atc act tgt cgg gcg agt      144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

cag ggt att agc agc tgg tta gcc tgg tat cag cag aaa cca gag aaa      192
Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

gcc cct aag tcc ctg atc tat gct gca tcc agt ttg caa agt ggg gtc      240
Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

cca tca agg ttc agc ggc agt gga tct ggg aca gat ttc act ctc acc      288
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

atc agc agc ctg cag cct gaa gat ttt gca act tat tac tgc caa cag      336
Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

tat aat agt tac ccg tac act ttt ggc cag ggg acc aag ctg gag atc      384
Tyr Asn Ser Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
        115                 120                 125

aaa cgt                                                             390
Lys Arg
    130


<210>  65
<211>  130
<212>  PRT
<213>  Homo sapiens

<400>  65

Met Asp Met Arg Val Leu Ala Gln Leu Leu Gly Leu Leu Leu Leu Cys
1               5                   10                  15
```


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```
Phe Pro Gly Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
        35                  40                  45

Gln Gly Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys
    50                  55                  60

Ala Pro Lys Ser Leu Ile Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            100                 105                 110

Tyr Asn Ser Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile
        115                 120                 125

Lys Arg
    130
```

<210> 66
<211> 351
<212> DNA
<213> Artificial

<220>
<223> HV0 Mouse8213 VH

<220>
<221> CDS
<222> (1)..(351)

<400> 66

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cag gtc caa ctg gtg cag tct ggg gct gaa gtg aag aag cct ggg gct       48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                   10                  15

tca gtg aag gtg tcc tgc aag gct tct ggc tac acc ttc acc agc tac       96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

tgg atg cac tgg gtg cgg cag gcg cct gga caa ggc ctt gag tgg atg      144
Trp Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

gga gag att aat cct agc aac ggt cgt act aac tac aat gag aag ttc      192
Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe
    50                  55                  60
```


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```
aag acc cgg gtc aca atc act gca gac aca tcc acc agc aca gcc tac      240
Lys Thr Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

atg gaa ctc agc agc ctg cga tct gag gac act gcg gtc tat tac tgt      288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gcg cgg ggt tac tac ctc tac ttt gac tac tgg ggc caa ggc acc ctg      336
Ala Arg Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu
            100                 105                 110

gtc aca gtc tcc tca                                                  351
Val Thr Val Ser Ser
        115
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<210> 67
<211> 117
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 67

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Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                   10                  15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Trp Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

Gly Glu Ile Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn Glu Lys Phe
    50                  55                  60

Lys Thr Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala 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 Gly Tyr Tyr Leu Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu
            100                 105                 110

Val Thr Val Ser Ser
        115
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<210> 68
<211> 321
<212> DNA

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<213> Artificial

<220>
<223> LV0 Mouse8213 VL

<220>
<221> CDS
<222> (1)..(321)

<400> 68
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gac atc cag atg acc caa tct cca tcc tcc ttg tct gca tct gtg gga       48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

gac cga gtc acc atc act tgc cat gca agt cag ggc att agg att aat       96
Asp Arg Val Thr Ile Thr Cys His Ala Ser Gln Gly Ile Arg Ile Asn
            20                  25                  30

ata ggg tgg tat cag cag aag cca ggg aaa gca cct aag ctc ctg atc      144
Ile Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

tat cat gga acc aac ttg gaa gat gga gtt cca tca agg ttc agt ggc      192
Tyr His Gly Thr Asn Leu Glu Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

agt gga tct gga aca gat ttc act ctc acc atc agc agc ctg caa cct      240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

gaa gat ttt gca acc tat tac tgt gta cag tat ggt cag ttt ccg tgg      288
Glu Asp Phe Ala Thr Tyr Tyr Cys Val Gln Tyr Gly Gln Phe Pro Trp
                85                  90                  95

acg ttc ggt cag ggc acc aag ctg gaa atc aaa                         321
Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 69
<211> 107
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 69

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys His Ala Ser Gln Gly Ile Arg Ile Asn
            20                  25                  30

Ile Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr His Gly Thr Asn Leu Glu Asp Gly Val Pro Ser Arg Phe Ser Gly
```


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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 Val Gln Tyr Gly Gln Phe Pro Trp
85 90 95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 70
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Fw3

<400> 70
ctacacagag ctgtccttgg at 22

<210> 71
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Re3

<400> 71
tttctcagtg gctgtggtca 20

<210> 72
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Fw4NotI

<400> 72
aattgcggcc gccaccatgt tttcaggtct tac 33

<210> 73
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Primer mTim-3 Re4NotI

<400> 73
aattgcggcc gctcaggatg gctgctggct 30

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<210> 74
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mIgV_vecR1

<400> 74
ggaccttgta agtagtagca 20

<210> 75
<211> 22
<212> DNA
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<220>
<223> Primer hTIM3+mIgV_vecF1

<400> 75
gccaaggtca cccctgcacc ga 22

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<211> 35
<212> DNA
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<220>
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ctacttacaa ggtccttgga agatggttat aaggt 35

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<220>
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aggggtgacc ttggctgctt tgatgtctaa tttca 35

<210> 78
<211> 909
<212> DNA
<213> Artificial

<220>
<223> IgV chimeraTIM-3/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 78
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48

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```
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc ttg gaa gat ggt tat aag gtt gag gtt ggt aaa   96
Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30

aat gcc tat ctg ccc tgc agt tac act cta cct aca tct ggg aca ctt  144
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

gtg cct atg tgc tgg ggc aag gga ttc tgt cct tgg tca cag tgt acc  192
Val Pro Met Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

aat gag ttg ctc aga act gat gaa aga aat gtg aca tat cag aaa tcc  240
Asn Glu Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

agc aga tac cag cta aag ggc gat ctc aac aaa gga gat gtg tct ctg  288
Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

atc ata aag aat gtg act ctg gat gac cat ggg acc tac tgc tgc agg  336
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Thr Tyr Cys Cys Arg
            100                 105                 110

ata cag ttc cct ggt ctt atg aat gat aaa aaa tta gaa ctg aaa tta  384
Ile Gln Phe Pro Gly Leu Met Asn Asp Lys Lys Leu Glu Leu Lys Leu
        115                 120                 125

gac atc aaa gca gcc aag gtc acc cct gca ccg act cgg cag aga gac  432
Asp Ile Lys Ala Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca  480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa  528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac  576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca  624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att  672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc  720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca  768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255
```


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```
gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  79
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  79

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

Val Pro Met Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

Asn Glu Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Thr Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Phe Pro Gly Leu Met Asn Asp Lys Lys Leu Glu Leu Lys Leu
        115                 120                 125

Asp Ile Lys Ala Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160
```


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Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
165 170 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
180 185 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
195 200 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
210 215 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225 230 235 240

Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
245 250 255

Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
260 265 270

Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
275 280 285

Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 80
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_vecR2

<400> 80
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<210> 81
<211> 23
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_vecF2

<400> 81
ataggcatct acatcggagc agg 23

<210> 82
<211> 35
<212> DNA

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<213> Artificial

<220>
<223> Primer hTIM3+mMucin_insF2

<400> 82
ttggtcatca aaccagccaa ggtcactcca gctca 35

<210> 83
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3+mMucin_insR2

<400> 83
gatgtagatg cctattctga tcgtttctcc agagt 35

<210> 84
<211> 876
<212> DNA
<213> Artificial

<220>
<223> Mucin chimeraTIM-3/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(876)

<400> 84
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

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caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc      384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

atc aaa cca gcc aag gtc act cca gct cag act gcc cat ggg gac tct      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Gln Thr Ala His Gly Asp Ser
    130                 135                 140

act aca gct tct cca aga acc cta acc acg gag aga aat ggt tca gag      480
Thr Thr Ala Ser Pro Arg Thr Leu Thr Thr Glu Arg Asn Gly Ser Glu
145                 150                 155                 160

aca cag aca ctg gtg acc ctc cat aat aac aat gga aca aaa att tcc      528
Thr Gln Thr Leu Val Thr Leu His Asn Asn Asn Gly Thr Lys Ile Ser
                165                 170                 175

aca tgg gct gat gaa att aag gac tct gga gaa acg atc aga ata ggc      576
Thr Trp Ala Asp Glu Ile Lys Asp Ser Gly Glu Thr Ile Arg Ile Gly
            180                 185                 190

atc tac atc gga gca ggg atc tgt gct ggg ctg gct ctg gct ctt atc      624
Ile Tyr Ile Gly Ala Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile
        195                 200                 205

ttc ggc gct tta att ttc aaa tgg tat tct cat agc aaa gag aag ata      672
Phe Gly Ala Leu Ile Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile
    210                 215                 220

cag aat tta agc ctc atc tct ttg gcc aac ctc cct ccc tca gga ttg      720
Gln Asn Leu Ser Leu Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu
225                 230                 235                 240

gca aat gca gta gca gag gga att cgc tca gaa gaa aac atc tat acc      768
Ala Asn Ala Val Ala Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr
                245                 250                 255

att gaa gag aac gta tat gaa gtg gag gag ccc aat gag tat tat tgc      816
Ile Glu Glu Asn Val Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys
            260                 265                 270

tat gtc agc agc agg cag caa ccc tca caa cct ttg ggt tgt cgc ttt      864
Tyr Val Ser Ser Arg Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe
        275                 280                 285

gca atg cca tag                                                      876
Ala Met Pro
    290
```

<210> 85
<211> 291
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 85

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15
```


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```
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Gln Thr Ala His Gly Asp Ser
    130                 135                 140

Thr Thr Ala Ser Pro Arg Thr Leu Thr Thr Glu Arg Asn Gly Ser Glu
145                 150                 155                 160

Thr Gln Thr Leu Val Thr Leu His Asn Asn Asn Gly Thr Lys Ile Ser
                165                 170                 175

Thr Trp Ala Asp Glu Ile Lys Asp Ser Gly Glu Thr Ile Arg Ile Gly
            180                 185                 190

Ile Tyr Ile Gly Ala Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile
        195                 200                 205

Phe Gly Ala Leu Ile Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile
    210                 215                 220

Gln Asn Leu Ser Leu Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu
225                 230                 235                 240

Ala Asn Ala Val Ala Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr
                245                 250                 255

Ile Glu Glu Asn Val Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys
            260                 265                 270
```


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Tyr Val Ser Ser Arg Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe
275 280 285

Ala Met Pro
290

<210> 86
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47F1

<400> 86
acatctggga cacttgtgcc cgtctgctgg ggcaa 35

<210> 87
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R1

<400> 87
ataaccatct tccaaggacc ttgtaagtag tagca 35

<210> 88
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47F2

<400> 88
ctgcagttac actctaccta catctgggac acttg 35

<210> 89
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R2

<400> 89
ttaccaacct caaccttata accatcttcc aagga 35

<210> 90
<211> 35
<212> DNA
<213> Artificial

<220>

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<223> Primer hTIM3chimera22-47F3

<400> 90
aaatgcctat ctgccctgca gttacactct accta 35

<210> 91
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera22-47R3

<400> 91
ggcagatagg cattttacc aacctcaacc tta 33

<210> 92
<211> 906
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 22-47/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(906)

<400> 92
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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc ttg gaa gat ggt tat aag gtt gag gtt ggt aaa 96
Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
20 25 30

aat gcc tat ctg ccc tgc agt tac act cta cct aca tct ggg aca ctt 144
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc 288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
85 90 95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc 336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
100 105 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc 384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
115 120 125

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atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca      480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata      528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
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<210> 93
<211> 301
<212> PRT
<213> Artificial

<220>
<223> Synthetic Construct

<400> 93

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Leu Glu Asp Gly Tyr Lys Val Glu Val Gly Lys
            20                  25                  30
```


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```
Asn Ala Tyr Leu Pro Cys Ser Tyr Thr Leu Pro Thr Ser Gly Thr Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285
```


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Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 94
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera57-66F

<400> 94
acagtgtacc aatgaggtgc tcaggactga tgaaa 35

<210> 95
<211> 33
<212> DNA
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<220>
<223> Primer hTIM3chimera57-66R

<400> 95
gaccaaggac agaatccttt gccccagcag acg 33

<210> 96
<211> 906
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 57-66/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(906)

<400> 96
atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta 48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga ttc tgt cct tgg tca cag tgt acc 192
Val Pro Val Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
50 55 60

aat gag gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Glu Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

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aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg acc      288
Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc      336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc      384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca      480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata      528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
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<210> 97
<211> 301
<212> PRT
<213> Artificial

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<220>
<223> Synthetic Construct

<400> 97

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Phe Cys Pro Trp Ser Gln Cys Thr
    50                  55                  60

Asn Glu Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80

Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Thr
                85                  90                  95

Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240
```


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Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
245 250 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
260 265 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
275 280 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
290 295 300

<210> 98
<211> 34
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera67-105F

<400> 98
ctggatgacc atgggatcta ctgctgccgg atcc 34

<210> 99
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera67-105R

<400> 99
atcagttctg agcaacacgt tgccacattc aaa 33

<210> 100
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTIM3chimera67-105F

<400> 100
ttgctcagaa ctgatgaaag 20

<210> 101
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer mTIM3chimera67-105R

<400> 101

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cccatggtca tccagagtca 20

<210> 102
<211> 909
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 67-105/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 102

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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag       96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc      144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc      192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

aac gtg ttg ctc aga act gat gaa aga aat gtg aca tat cag aaa tcc      240
Asn Val Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

agc aga tac cag cta aag ggc gat ctc aac aaa gga gat gtg tct ctg      288
Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95

atc ata aag aat gtg act ctg gat gac cat ggg atc tac tgc tgc cgg      336
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Ile Tyr Cys Cys Arg
            100                 105                 110

atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg      384
Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac      432
Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca      480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa      528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac      576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
```


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Page 1352

```
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca      624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att      672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc      720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca      768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  103
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  103

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Leu Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Gln Leu Lys Gly Asp Leu Asn Lys Gly Asp Val Ser Leu
                85                  90                  95
```


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Page 1353

```
Ile Ile Lys Asn Val Thr Leu Asp Asp His Gly Ile Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300

<210>  104
<211>  35
<212>  DNA
<213>  Artificial

<220>
<223>  Primer hTIM3chimera74-81F

<400>  104
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gtgacatatc agaaatcctc cagatactgg ctaaa 35

<210> 105
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera74-81R

<400> 105
tttctgatat gtcacattcc tttcatcagt cctga 35

<210> 106
<211> 909
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 74-81/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(909)

<400> 106

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atg ttt tca cat ctt ccc ttt gac tgt gtc ctg ctg ctg ctg ctg cta   48
Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                  10                  15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag   96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc  144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc  192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

aac gtg gtg ctc agg act gat gaa agg aat gtg aca tat cag aaa tcc  240
Asn Val Val Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

tcc aga tac tgg cta aat ggg gat ttc cgc aaa gga gat gtg tcc ctg  288
Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu
                85                  90                  95

acc ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg  336
Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg
            100                 105                 110

atc caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg  384
Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

gtc atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac  432
Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
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```
    130                 135                 140

ttc act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca      480
Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

gca gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa      528
Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

ata tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac      576
Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

tta cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca      624
Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

ggg atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att      672
Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

ttc aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc      720
Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

atc tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca      768
Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

gag gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta      816
Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

tat gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg      864
Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

cag caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag          909
Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300


<210>  107
<211>  302
<212>  PRT
<213>  Artificial

<220>
<223>  Synthetic Construct

<400>  107

Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1               5                   10                  15

Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30

Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
        35                  40                  45
```


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Page 1356

```
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
    50                  55                  60

Asn Val Val Leu Arg Thr Asp Glu Arg Asn Val Thr Tyr Gln Lys Ser
65                  70                  75                  80

Ser Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu
                85                  90                  95

Thr Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg
            100                 105                 110

Ile Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu
        115                 120                 125

Val Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp
    130                 135                 140

Phe Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro
145                 150                 155                 160

Ala Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln
                165                 170                 175

Ile Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp
            180                 185                 190

Leu Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala
        195                 200                 205

Gly Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile
    210                 215                 220

Phe Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu
225                 230                 235                 240

Ile Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala
                245                 250                 255

Glu Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val
            260                 265                 270

Tyr Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg
        275                 280                 285

Gln Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
```


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290 295 300

<210> 108
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera88-96F

<400> 108
aaaggagatg tgtctctgat catagagaat gtgac 35

<210> 109
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Primer hTIM3chimera88-96R

<400> 109
agacacatct cctttgttga gatccccatt tagcc 35

<210> 110
<211> 906
<212> DNA
<213> Artificial

<220>
<223> TIM-3 chimera 88-96/pEF6 Myc_HisC

<220>
<221> CDS
<222> (1)..(906)

<400> 110
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Met Phe Ser His Leu Pro Phe Asp Cys Val Leu Leu Leu Leu Leu Leu
1 5 10 15

cta ctt aca agg tcc tca gaa gtg gaa tac aga gcg gag gtc ggt cag 96
Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
20 25 30

aat gcc tat ctg ccc tgc ttc tac acc cca gcc gcc cca ggg aac ctc 144
Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
35 40 45

gtg ccc gtc tgc tgg ggc aaa gga gcc tgt cct gtg ttt gaa tgt ggc 192
Val Pro Val Cys Trp Gly Lys Gly Ala Cys Pro Val Phe Glu Cys Gly
50 55 60

aac gtg gtg ctc agg act gat gaa agg gat gtg aat tat tgg aca tcc 240
Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65 70 75 80

aga tac tgg cta aat ggg gat ctc aac aaa gga gat gtg tct ctg atc 288
Arg Tyr Trp Leu Asn Gly Asp Leu Asn Lys Gly Asp Val Ser Leu Ile

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                85                  90                  95

ata gag aat gtg act cta gca gac agt ggg atc tac tgc tgc cgg atc      336
Ile Glu Asn Val Thr Leu Ala Asp Ser Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110

caa atc cca ggc ata atg aat gat gaa aaa ttt aac ctg aag ttg gtc      384
Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
        115                 120                 125

atc aaa cca gcc aag gtc acc cct gca ccg act cgg cag aga gac ttc      432
Ile Lys Pro Ala Lys Val Thr Pro Ala Pro Thr Arg Gln Arg Asp Phe
    130                 135                 140

act gca gcc ttt cca agg atg ctt acc acc agg gga cat ggc cca gca      480
Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
145                 150                 155                 160

gag aca cag aca ctg ggg agc ctc cct gat ata aat cta aca caa ata      528
Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
                165                 170                 175

tcc aca ttg gcc aat gag tta cgg gac tct aga ttg gcc aat gac tta      576
Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
            180                 185                 190

cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
        195                 200                 205

atc tgt gct ggg ctg gct ctg gct ctt atc ttc ggc gct tta att ttc      672
Ile Cys Ala Gly Leu Ala Leu Ala Leu Ile Phe Gly Ala Leu Ile Phe
    210                 215                 220

aaa tgg tat tct cat agc aaa gag aag ata cag aat tta agc ctc atc      720
Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

tct ttg gcc aac ctc cct ccc tca gga ttg gca aat gca gta gca gag      768
Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
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gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
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Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
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Page 1360

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65                  70                  75                  80

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ata aag aat gtg act ctg gat gac cat ggg atc tac tgc tgc cgg atc      336
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cgg gac tct gga gca acc atc aga ata ggc atc tac atc gga gca ggg      624
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        195                 200                 205

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Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

gga att cgc tca gaa gaa aac atc tat acc att gaa gag aac gta tat      816
Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

gaa gtg gag gag ccc aat gag tat tat tgc tat gtc agc agc agg cag      864
Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

caa ccc tca caa cct ttg ggt tgt cgc ttt gca atg cca tag              906
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Leu Leu Thr Arg Ser Ser Glu Val Glu Tyr Arg Ala Glu Val Gly Gln
            20                  25                  30


Asn Ala Tyr Leu Pro Cys Phe Tyr Thr Pro Ala Ala Pro Gly Asn Leu
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Asn Val Val Leu Arg Thr Asp Glu Arg Asp Val Asn Tyr Trp Thr Ser
65                  70                  75                  80


Arg Tyr Trp Leu Asn Gly Asp Phe Arg Lys Gly Asp Val Ser Leu Ile
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Ile Lys Asn Val Thr Leu Asp Asp His Gly Ile Tyr Cys Cys Arg Ile
            100                 105                 110


Gln Ile Pro Gly Ile Met Asn Asp Glu Lys Phe Asn Leu Lys Leu Val
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Thr Ala Ala Phe Pro Arg Met Leu Thr Thr Arg Gly His Gly Pro Ala
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Glu Thr Gln Thr Leu Gly Ser Leu Pro Asp Ile Asn Leu Thr Gln Ile
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Ser Thr Leu Ala Asn Glu Leu Arg Asp Ser Arg Leu Ala Asn Asp Leu
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Arg Asp Ser Gly Ala Thr Ile Arg Ile Gly Ile Tyr Ile Gly Ala Gly
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          195                 200                 205

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    210                 215                 220

Lys Trp Tyr Ser His Ser Lys Glu Lys Ile Gln Asn Leu Ser Leu Ile
225                 230                 235                 240

Ser Leu Ala Asn Leu Pro Pro Ser Gly Leu Ala Asn Ala Val Ala Glu
                245                 250                 255

Gly Ile Arg Ser Glu Glu Asn Ile Tyr Thr Ile Glu Glu Asn Val Tyr
            260                 265                 270

Glu Val Glu Glu Pro Asn Glu Tyr Tyr Cys Tyr Val Ser Ser Arg Gln
        275                 280                 285

Gln Pro Ser Gln Pro Leu Gly Cys Arg Phe Ala Met Pro
    290                 295                 300
```

Claims

1. A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (i) to (iii):

   (i) an antibody which comprises an H chain of an antibody which comprises complementary determining regions CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
   (ii) an antibody which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
   (iii) an antibody which comprises an H chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

2. The monoclonal antibody or the antibody fragment thereof according to claim 1, wherein the monoclonal antibody binds to the same epitope to which one antibody selected from the above (i) to (iii) binds.

3. A monoclonal antibody or an antibody fragment thereof, which binds to an extracellular region of human TIM-3 while competing with one antibody selected from the following (a) and (b):

   (a) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
   (b) an antibody which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

4. The monoclonal antibody or the antibody fragment thereof according to claim 3, which binds to the same epitope to which one antibody selected from the above (a) or (b) binds.

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5. The monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 4, which is a recombinant antibody.

6. The monoclonal antibody or the antibody fragment thereof according to claim 5, which is a recombinant antibody selected from a human chimeric antibody, a humanized antibody and a human antibody.

7. A monoclonal antibody or an antibody fragment thereof, which is one selected from the following (i) to (iii):

(i) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 1 to 3, respectively, and comprises an L chain of an antibody which comprises CDRs1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 4 to 6, respectively,
(ii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 11 to 13, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 14 to 16, respectively,
(iii) a monoclonal antibody and an antibody fragment thereof which comprises an H chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 21 to 23, respectively, and comprises an L chain of an antibody which comprises CDRs 1 to 3 comprising the amino acid sequences represented by SEQ ID NOs: 24 to 26, respectively.

8. A monoclonal antibody and an antibody fragment thereof, which is one selected from the following (a) and (b):

(a) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 8 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 10,
(b) a monoclonal antibody and an antibody fragment thereof which comprises VH of an antibody comprising the amino acid sequence represented by SEQ ID NO: 18 and comprises VL of an antibody comprising the amino acid sequence represented by SEQ ID NO: 20.

9. A monoclonal antibody and an antibody fragment thereof which binds to the amino acid sequences at positions 67 to 105, the amino acid sequences at positions 67 to 96 or the amino acid sequences at positions 67 to 87 in the amino acid sequence of IgV domain of human TIM-3 represented by SEQ ID NO: 53.

10. The antibody fragment according to any one of claims 1 to 9, wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region (diabody), a disulfide stabilized V region (dsFv) and a peptide comprising CDR.

11. A DNA which encodes the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

12. A recombinant vector which comprises the DNA according to claim 11.

13. A transformant obtainable by introducing the recombinant vector according to claim 12 into a host cell.

14. A process for producing the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10, which comprises culturing the transformant according to claim 13 in culture to form and accumulate the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10, and recovering the monoclonal antibody or the antibody fragment thereof from the culture.

15. A method for immunologically detecting or measuring a human TIM-3 which comprises using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

16. A reagent for detecting or measuring a human TIM-3 which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

17. A diagnostic agent for a disease relating to a human TIM-3 positive cell which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

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18. A method for diagnosing a disease relating to a human TIM-3 positive cell which comprises detect or measure a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

19. A method for diagnosing a disease relating to a human TIM-3 positive cell, which comprises ditecting or measuring a human TIM-3 using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

20. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10 for the manufacture of a diagnostic agent for a disease relating to a human TIM-3 positive cell.

21. A therapeutic agent for a disease relating to a human TIM-3 positive cell, which comprises the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

22. A therapeutic method for a disease relating to a human TIM-3 positive cell, which comprises inducing cell death of a human TIM-3 positive cell using the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10.

23. Use of the monoclonal antibody or the antibody fragment thereof according to any one of claims 1 to 10 for the manufacture of a therapeutic agent for a disease relating to a human TIM-3 positive cell.

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Fig. 1

	1	2	3	4	5	6
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
8213 antibody HV0	QVQLVQSGAE	VKKPGASVKV	SCKASGYTFT	SYWMHWVRQA	PGQGLEWMGE	INPSNGRTNY
8213 antibody HV3				K	I	
8213 antibody HV4				K	I	
8213 antibody HV5				K R	I	
8213 antibody HV6		V				
8213 antibody HV7				K R	I	
8213 antibody HV8		L		K	I	
8213 antibody HV10		V		K R	I	
8213 antibody HV12		V L		K R	I	

	7	8	9	10	11	
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567
8213 antibody HV0	NEKFKTRVTI	TADTSTSTAY	MELSSLRSED	TAVYYCARGY	YLYFDYWGQG	TLVTVSS
8213 antibody HV3				G		
8213 antibody HV4	K	K				
8213 antibody HV5	K			G		
8213 antibody HV6	KA	V K		G		
8213 antibody HV7	K	V K		G		
8213 antibody HV8	A L	V		G	L	
8213 antibody HV10	KA L	V K		G		
8213 antibody HV12	KA L	V K		G		

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Fig. 2

	1	2	3	4	5	6
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
8213 antibody LV0	DIQMTQSPSS	LSASVGDRVT	ITCHASQGIR	INIGWYQQKP	GKAPKLLIYH	GTNLEDGVPS
8213 antibody LV2				L	F	
8213 antibody LV4		L		L	G	
8213 antibody LV5				L	SF G	
8213 antibody LV6		L		L	F G	
8213 antibody LV7		M L		L	F G	
8213 antibody LV9		M V L		L	SF G	

	7	8	9	10	
	1234567890	1234567890	1234567890	1234567890	1234567
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8213 antibody LV2					
8213 antibody LV4		Y			
8213 antibody LV5			D		
8213 antibody LV6		Y	D		
8213 antibody LV7		Y	D		
8213 antibody LV9		Y	D		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

A. CLASSIFICATION OF SUBJECT MATTER
A61P7/00(2006.01)i, *A61K39/00*(2006.01)i, *C07K16/28*(2006.01)i, *C12N1/15*(2006.01)i, *C12N1/19*(2006.01)i, *C12N1/21*(2006.01)i, *C12N5/10*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/08*(2006.01)i
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P7/00, A61K39/00, C07K16/28, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N15/09, C12P21/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2011
Kokai Jitsuyo Shinan Koho 1971-2011 Toroku Jitsuyo Shinan Koho 1994-2011

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubMed, JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2010-510223 A (THE BRIGHAM AND WOMEN'S HOSPITAL, INC.), 02 April 2010 (02.04.2010), & US 2010/0061992 A1 & EP 2081961 A2 & WO 2008/060617 A2 & CA 2668693 A1	1-17,20,21, 23
Y	JP 2005-526018 A (THE BRIGHAM AND WOMEN'S HOSPITAL, INC., DANA-FARBER CANCER INSTITUTE, INC.), 02 September 2005 (02.09.2005), & US 2004/0005322 A1 & EP 1467759 A2 & WO 2003/063792 A2	1-17,20,21, 23
Y	JP 2007-530560 A (TELOS PHARMACEUTICALS, INC.), 01 November 2007 (01.11.2007), & US 2005/0276756 A1 & EP 1740224 A2 & WO 2005/097211 A2 & KR 10-2007-0037570 A	1-17,20,21, 23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 September, 2011 (06.09.11)	13 September, 2011 (13.09.11)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MAJETI, R. et al., Dysregulated gene expression networks in human acute myelogenous leukemia stem cells., Proc. Natl. Acad. Sci. USA., 2009, Vol.106, No.9, p.3396-3401	1-17,20,21, 23
Y	IIDA S., et al., Nonfucosylated Therapeutic IgG1 Antibody Can Evade the Inhibitory Effect of Serum Immunoglobulin G on Antibody-Dependent Cellular Cytotoxicity through its High Binding to FcγRIIIa., Clin. Cancer Res., 2006, Vol.12, No.9, p.2879-2887	1-17,20,21, 23
Y	WO 2002/031140 A1 (KYOWA HAKKO KOGYO CO., LTD.), 18 April 2002 (18.04.2002), & US 2003/0115614 A1 & EP 1331266 A1 & WO 2002/031140 A1 & CN 1894406 A	1-17,20,21, 23
Y	WO 2000/061739 A1 (KYOWA HAKKO KOGYO CO., LTD.), 19 October 2000 (19.10.2000), & US 2005/0272916 A1 & EP 1176195 A1 & WO 2000/061739 A1	1-17,20,21, 23
P,Y	WO 2010/117057 A1 (KYOWA HAKKO KIRIN CO., LTD.), 14 October 2010 (14.10.2010), (Family: none)	1-17,20,21, 23
A	ANDERSON, A.C. et al., Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells., Science, 2007, Vol.318, No.5853, p.1141-1143	1-17,20,21, 23
A	FUKUSHIMA, A. et al., Antibodies to T-cell Ig and mucin domain-containing proteins(Tim)-1 and -3 suppress the induction and progression of murine allergic conjunctivitis., Biochem. Biophys. Res. Commun., 2007, Vol.353 No.1, p.211-216	1-17,20,21, 23
A	JU, Y. et al., T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) mediates natural killer cell suppression in chronic hepatitis B., J. Heapatol., Vol.52, No.3, 2010.01.06, p.322-329	1-17,20,21, 23
A	HASTINGS W.D., TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines., Eur. J. Immunol., 2009, Vol.39, No.9, p.2492-2501	1-17,20,21, 23

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/063396

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18,19,22
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 18, 19 and 22 pertain to diagnostic methods to be practiced on the human body or methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under the provisions of PCT Rule 39.1(iv), to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 9627603 A **[0010]**
- WO 2003063792 A **[0010]**
- WO 2009091547 A **[0010]**
- JP 61178926 A **[0125]**
- JP 56023587 A **[0125]**
- JP 2117920 A **[0125]**
- JP 58110600 A **[0164]**
- JP 60221091 A **[0164]**
- US 4686191 A **[0164]**
- US 4939094 A **[0164]**
- US 5160735 A **[0164]**
- JP 3022979 A **[0168]**
- JP 2227075 A **[0168] [0171] [0179]**
- WO 9710354 A **[0168] [0210]**
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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

15 February 2016

Dear Sirs

European Patent Application No. 11792564.4-1412
KYOWA HAKKO KIRIN CO., LTD. *et al.*
Our Ref: N118545-EP PCT/cxe

In response to the Communication pursuant to Article 94(3) EPC dated 8 October 2015, please amend this application by replacing the claims presently on file with the new claims provided herewith. For the Examiner's assistance a track-changes copy of the claims showing the amendments being made to them is also provided.

Please also replace the sequence listing currently on file with the corrected sequence listing provided herewith. A hand-amended copy of original sequence listing pages 3 to 9 is also attached to show the corrections being made.

A copy of the pages of Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) showing the signal peptides of human kappa light chains and the signal peptides of human heavy chains is attached as evidence of the common general knowledge at the priority date.

An Experimental Report as referred to below is attached.

I am also filing an English translation of the priority document in view of the reference made below to D11, which was cited in categories X and P in the extended European Search Report.

Amendments

Claims 1, 3 and 5 have been amended to specify that the antibody exhibits ADCC activity. This amendment finds basis in the originally filed application at, for example, page 3 lines 11 to 14.

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Inventive Step

The claimed antibodies involve an inventive step. As disclosed at page 3, paragraph 12 of the application, an object of the present invention is to provide a monoclonal antibody or an antibody fragment which binds to the amino acid sequence or the three-dimensional structure of the extracellular region of TIM-3 and expresses ADCC activity.

None of the cited documents disclose an anti-TIM-3 monoclonal antibody having ADCC activity. The present inventors have made the unexpected finding that antibodies binding to a particular epitope in the extracellular domain of TIM-3 have good ADCC activity, whilst antibodies binding to other epitopes do not. The ADCC activity of the antibodies of the invention is therefore linked to their binding to a particular epitope in TIM-3.

As demonstrated in the present application, the anti-TIM-3 monoclonal antibodies produced in Examples 12 to 14 (i.e., antibodies 512, 644, 4545 and 4177) and the anti-TIM-3 monoclonal antibody 8213 bind to IgV domain in the extracellular domain of TIM-3 (see paragraph [0405] of the present application). Further, the identification by the present inventors of the specific binding site in the IgV domain revealed that the antibodies 4545, 4177, and 8213 which bind to the amino acid sequence at positions 67 to 105 of the IgV domain (see paragraph [0425] and [0426] of the present application) have ADCC activity (see paragraphs [0371] and [0375] of the present application).

As explained hereinafter, the ADCC activity exhibited by binding of antibodies 4545 and 4177 of the present invention to the extracellular domain of TIM-3 is dependent on the epitope in TIM-3 and not on the binding activities of these antibodies to the antigen.

The Figure in the attached Experimental Report demonstrates that the anti-TIM-3 monoclonal antibodies 512, 644, 4545, and 4177 have almost equivalent binding activities to TIM-3 expressing HEK293 cells.

Further, the Table in the attached Experimental Report shows that an anti-dinitrophenol (DNP) antibody which was used as negative control exhibited almost no ADCC activity to Daudi cells, while the anti-TIM-3 monoclonal antibodies 4545 and 4177 exhibited significantly higher ADCC activity (see also paragraphs [0371] and [0375] of the present application). On the other hand, the anti-TIM-3 monoclonal antibodies 512 and 644 which bind to different epitopes from the antibodies 4545 and 4177 exhibited almost no ADCC activity.

The results shown in the attached Experimental Report reveal that although the anti-TIM-3 monoclonal antibodies 512, 644, 4545, and 4177 have almost equivalent binding activities to TIM-3, the strength of ADCC activity is different between the antibodies 4545 and 4177 versus the antibodies 512 and 644. This difference in the strength of ADCC activity is attributable to the binding of the 4545 and 4177 antibodies to a different epitope to those of the 512 and 644 antibodies.

Further, Kikushige *et al.* cited as D11 in the extended European Search Report shows that the mouse monoclonal antibody 8213 ("ATIK2a") specifically binds to TIM-3 expressing cells and has ADCC activity (see Figures 5A and 5B of D11). Therefore, the antibodies 4545, 4177 and 8213 of the present invention bind to the specific epitopes in the extracellular domain of TIM-3 and exhibit ADCC activity.

D1 teaches that based on the expression or the function of TIM-3 molecule, an agent affecting TIM-3 activity (including an anti-TIM-3 antibody) may be an antagonist antibody that blocks the binding interactions between TIM-3 and TIM-3 ligands or an agonist antibody that triggers TIM-3 intracellular signaling (see WO08/060617, "C. antibodies" beginning on page 35, first and second paragraphs; and "D. Method to determine effect on TIM-3 activity" beginning on page 38, first and second paragraphs). D1 also teaches that the antibody may bind to the IgV domain in the extracellular domain of TIM-3 (claim 10 of D1). However, D1 does not teach or suggest that ADCC activity of an antibody binding TIM-3 may be a useful property. Nor is there anything in D1 to indicate that ADCC activity of an anti-TIM-3 antibody may depend on the epitope to which it binds.

D2 discloses the specific anti-TIM-3 antibodies 8B.2C12 and 25F.1D6 which were obtained by immunizing rats (WO 03/063792, page 3, lines 20-22; and Example 1). Like D1, D2 does not teach or suggest that ADCC activity of an antibody binding TIM-3 may be a useful property or indicate that ADCC activity of an anti-TIM-3 antibody may depend on the epitope to which it binds.

D3 teaches that the agent can be a chimeric polypeptide that includes a TIM polypeptide and a heterologous polypeptide such as the Fc region of the IgG antibody (WO 2005/097211, page 19, lines 16-19) and the Fc region is defined on page 20, first full paragraph. Further, D3 teaches that TIM-3 is expressed in lymphomas, thymomas, and mastocytomas (page 34, last paragraph; and Example XV). The Examiner considers that D3 contemplates that anti-TIM-3 antibodies may have ADCC activity. However, D3 does not describe ADCC activity of an anti-TIM-3 antibody. Therefore, the skilled person reading D3 would not know that ADCC activity of an antibody which binds to TIM-3 may depend on the specific region (epitope) in the extracellular (or IgV) domain of TIM-3 to which the antibody binds.

None of the cited prior art documents disclose an antibody binding to the same epitope on TIM-3 as the antibodies 4545, 4177 and 8213 or an anti-TIM-3 antibody having ADCC activity. Therefore, the objective technical problem in view of any of D1 to D3 may be defined as the provision of anti-TIM-3 antibodies with ADCC activity. This problem is solved by the present claims, which define antibodies binding to the same epitope as antibodies 4545, 4177 and 8213 and which have ADCC activity.

The skilled person would not have been motivated based on any of D1 to D3 to identify antibodies binding to epitopes in the TIM-3 IgV domain which are associated with ADCC activity. The skilled person would not have expected ADCC activity to be dependent on the epitope to which the antibodies bind. Accordingly, the present claims solve the problem of providing anti-TIM-3 antibodies with ADCC activity and the solution provided by the present claims would not have been obvious to the skilled person at the priority date.

Sufficiency

As described in Example 17, paragraphs [0371] and [0375] of the present application, the antibodies of the present invention have significantly high ADCC activity compared both to antibodies binding to different epitopes in the TIM-3 extracellular domain and to a control antibody that exhibited almost no ADCC activity to Daudi cells. Therefore, the antibodies of the present invention specifically bind to TIM-3 expressing cells and remove the same, and it is clear that the present invention is useful for treating the diseases caused by TIM-3 positive cells.

Correction of Sequence Listing

I request correction under Rule 139 EPC of SEQ ID NOs: 8, 10, 18 and 20 to remove the signal sequences that were erroneously included. It is immediately evident that an error has occurred and what the correction should be.

It would be immediately evident that inclusion of the signal sequences in SEQ ID NOs: 8, 10, 18 and 20 was unintentional. Claim 3 of the original application defines an antibody comprising a VH comprising SEQ ID NO: 8 and a VL comprising SEQ ID NO: 10 and an antibody comprising a VH comprising SEQ ID NO: 18 and a VL comprising SEQ ID NO: 20. The signal sequences of VH and VL chains are not present in an antibody because the sequences are cleaved off during antibody production in cells. Therefore, it is clearly an error that the signal sequences are present in each of SEQ ID NOs: 8, 10, 18 and 20.

Using his common general knowledge, the skilled person would have no difficulty determining which of the amino acids shown in each of SEQ ID NOs: 8, 10, 18 and 20 represent signal sequences.

As disclosed at page 67, paragraphs [0222] and [0223] of the present application, the presence of a signal sequence in a VL or VH amino acid sequence can be determined by comparing the length of the VL or VH amino acid sequence with those of known antibodies. As shown in the reference document attached hereto (Kabat et al., Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services (1991)), the signal sequences of SEQ ID NOs: 10 (VL), 18 (VH) and 20 (VL) are the same as those disclosed in Kabat et al., and the signal sequence of SEQ ID NO: 8 (VH) is almost the same as those disclosed in Kabat et al. except for only one residue (i.e., 11th Val is replaced with Ala).

In this regard, the signal sequences derived from known heavy and light chains, which are the same as (or almost the same as) those of SEQ ID NOs: 8, 10, 18 and 20, were the common technical knowledge as of the filing date. Thus, the ordinary skilled person would immediately recognise which parts of SEQ ID NOs: 8, 10, 18 and 20 represent the amino acid sequences of the signal sequences.

Accordingly, the correction of SEQ ID NOs: 8, 10, 18 and 20 to remove the signal sequences meets with the requirements of Rule 139 EPC.

Conclusion

Favourable reconsideration is respectfully requested in view of the amendments now made and the arguments set out above. If, however, there are any queries outstanding, please issue an Examination Report or telephone. Only as a precaution in case the Examiner is minded to refuse the application, I request Oral Proceedings.

In addition, if the Examiner is minded to issue a Rule 71(3) Communication but wishes to propose amendments to the claims or description, it is requested that the Examiner telephones the undersigned to discuss this before the Rule 71(3) Communication is issued. I look forward to hearing from the Examiner in due course.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH

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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

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Date
09-09-2019

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Acknowledgment of receipt of observations by third parties (Article 115 EPC)

Receipt of your letter dated 04.09.19 is hereby acknowledged.

Under Article 115 EPC you will not be a party to the proceedings before the European Patent Office.

☐ In your letter the following documents are mentioned which were not enclosed, and which are not available in the EPO:

☐ The third party observations have not been filed in an official language of the EPO (R. 114(1) EPC).

You are requested to file copy(ies) and/or translation(s) in one of the official EPO languages within **two months** of notification of this communication if they are to be taken into account.

For the Examining Division

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Application No.: 16 195 355.9

Decision of the Examining Division

In the oral proceedings held on 05.09.2019, the examining division has decided:

The European patent application is refused on the basis of Article 97(2) EPC. The reasons for the decision are attached (Form(s) 2916).

20.01.20
Date

Kley, Britta
Legal Member

Orlando, Michele
Chairman

Collura, Alessandra
1st examiner

Rodríguez-Palmero, M
2nd examiner

Enclosure(s): Form 2916

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Application No.	Ref.	Date
16 195 355.9 - 1112		07.02.2020
Applicant: Alexion Pharmaceuticals, Inc.		

Provision of a copy of the minutes in accordance with Rule 124(4) EPC

The attached copy of the minutes of the oral proceedings is sent to you in accordance with Rule 124(4) EPC.

Ruiz Fernandez, José
For the Examining Division
Tel. No.: +49 89 2399 - 7960

Enclosure(s): Copy of the minutes (Form 2009)

Registered Letter
EPO Form 2042 12.07TRI

After deliberation of the examining division, the chairman announced the following **decision** :

"The European patent application is refused."

Regarding the reasons for the decision, the chairman referred to:

Article 97(2) EPC: the application does not meet the requirements of Article/s R. 139, Art. 123(2), 76(1), 54 EPC.

The party/parties was/were informed that the minutes of the oral proceedings and a written decision (including an indication of the possibility of appeal) will be notified to him/them as soon as possible.

The chairman **closed the proceedings** on 05.09.2019 at 18:25 hours.

signed:

Orlando, Michele
. . . . . .
Chairman

signed:

Rodríguez-Palmero, M
. . . . . .
Minute Writer

Enclosure(s):
Annexes A-M

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Application No. : 16 195 355.9

Minutes of the oral proceedings before the EXAMINING DIVISION

The proceedings were not public.

Proceedings opened on 05.09.2019 at 09:10 hours

Examining Division:

Chairman:	Orlando, Michele
1st member:	Collura, Alessandra
2nd member:	Rodríguez-Palmero, M
Legal member:	Kley, Britta
Minute writer:	Rodríguez-Palmero, M

Present as/for the applicant/s:

TUXWORTH PM and SIMONS A as representatives
SPALDING TN and RAHEMBA TR, employees at Alexion Pharmaceuticals, Inc.

The identity of the person/s present and, where necessary, the authorisation to represent/authority to act were checked.

Essentials of the oral proceedings and relevant statements of the applicant/s:

As announced in the summons of 27-03-2019, for procedural economy, the applications 16195355.9 and 16172335.8, both divisional applications of 11001632.6, were dealt with sequentially in the oral proceedings of 05-09-2019. The minutes of the oral proceedings are thus written for both applications together.

Application 16195355.9

1 The applicant (A) confirmed the requests on file, namely the main request (MR) of 16-01-2018 and auxiliary requests (AR) 1-3 filed with the letter of 05-07-2019. The A further requested the possibility of filing further requests during the hearing.

2 The examining division (ED) pointed out that third party observations had been filed on 02-09-2019 (for application nr. 16195355.9), on 03-09-2019 (for application nr. 16172335.8) and on 04-09-2019 (for both applications). As the A had not received the latter, the proceedings were interrupted from 9:15 to 9:30 to provide the A with a copy.

3 The A then requested a further interruption to analyse the content of the last third party submission. For this purpose, the proceedings were interrupted between 9:30 and 10:15. After this break, the A asserted they had had enough time to examine its content.

4 As the **MR** on file was that referred to in the summons of 27-03-2019, the ED pointed to the objection under Art. 76(1) and 123(2) EPC raised therein. The A put forward their arguments. In particular, that the person skilled in the art would recognise that there was a signal peptide in the SEQ ID NO: 4 on p. 44 of the application, he would recognise its length with certainty and would know that it had to be removed for the purpose of the application. Therefore, the amendment in claim 1 of the MR was the obvious correction of an obvious error, allowable under Rule 139 EPC. In support for these arguments, the A referred to D14, items 8 and 11 and exhibits B, D and E attached thereto and also contested the arguments of the third party submissions of 04-09-2019, in particular those referring to the "Vink declaration" (attached thereto).

5 The proceedings were then interrupted for deliberation (10:40-11:00). The ED announced that the MR contravened R. 139, Art. 123(2) and 76(1) EPC. The C also pointed out that this appeared to apply also to **AR2**.

6 The A was then invited to provide the basis for the amendments in **AR1**. The A cited p. 4, l. 32 and the sequences on p. 44. The ED pointed to p 5, l. 30-33 and the interpretation of the scope of claim 1 in the light of the term "shown in" was

discussed. The A admitted that it was meant to include antibodies in which the light chain did not comprise the leading peptide.

7 After a break between 11:15 and 11:25, the ED announced that the AR1 did not fulfil the requirements of Art. 123(2) and 76(1) EPC. The C also pointed out that this appeared to apply also to **AR3**.

8 In order to facilitate the A with the preparation of a new request to overcome all objections mentioned in the summons against the MR, the ED pointed out that the objection under Art. 54 EPC mentioned in the summons for the MR also appeared to apply to AR1-3. The chairman of the ED gave the floor to the legal member of the ED to put forward the arguments in this matter. The extent of the confidentiality agreements used in the clinical trials described in D3 and D4 was discussed. Reference was made to the "Master clinical study agreement", item 7, and to the "Confidential disclosure agreement", item 2, both in D31. In particular, the legal member emphasized that the confidentiality agreement clearly excluded any information from the confidentiality obligation that had already become public. Since the sequence had been published by CAS with the intention to lay the structure open any signatory to the agreement must have understood it as excluding the sequence from confidentiality. Also, D1-D5 were cited. The A emphasized that the sequence published in CAS contained a mistake and the sequences mentioned in the claims (corresponding to the correct sequence of eculizumab) were not public before the priority date of the application and thus also not at the time at which the confidentiality agreements were signed. In this context, the A stated that "unfortunately a mistake was made in the publication of the sequence of eculizumab, although this was not our intention".

9 There was a break for deliberation between 11:55 and 12:30. Thereupon, the ED announced that the correct structure of eculizumab was considered to be available to the public before the priority date of the application. Therefore, the MR and AR1-3 were also considered to contravene Art. 54 EPC.

10 There was a lunch break between 12:30 and 13:30.

11 Upon re-opening, the A asked for adjournment of the oral proceedings. The reason provided by the A was that there was a new argument provided by the legal member concerning the confidentiality agreements. As these agreements were subject to national law, the A needed to consult experts in this matter.

12 After a break between 13:35-13:50, the discussion went on as to whether eculizumab had only been made available to the public in the context of the clinical trials.

13 The proceedings were again interrupted between 14:00 and 14:10. The ED announced that the request of postponement of the proceedings was not granted.

14 After a further break requested by the A between 14:15 and 14:30, the A stated that there were no further requests. The ED announced that the application was refused because none of the requests complied with the requirements of R. 139, Art. 123(2), 76(1) and 54 EPC.

Application 16172335.8

15 The proceedings were interrupted between 14:30 and 15:00 to provide the A with the corresponding third party observation for this application, which was filed on the 04-09-2019. The A was given time to analyse it. After opening the proceedings at 15:00, the A confirmed that they had enough time to consider this new submission and had no further comments on it.

16 The ED remarked that the objection under Art. 76(1) and 123(2) EPC raised in the summons concerning the exclusion of the signal peptide from the sequence mentioned in the claims applied to **MR**, **AR1** and **AR2** of 05-07-2019. The ED observed that the objection was identical to that discussed for the previous file (see items 4-7 above). Upon request from the ED, the A asserted that they had no new arguments concerning this matter. Thus, the ED announced that the MR, AR1 and AR2 did not comply with R. 139, Art. 76(1) and 123(2) EPC.

17 The A filed new AR3-6 containing handwritten amendments (see annexes A-D).

18 Concerning **AR3**, the A provided the basis in the original documents of the application, in particular p. 5, l. 30-33 and p. 19, l. 6-7. After short consultation the ED announced that the AR3 complied with Art. 123(2), 76(1) and 54 EPC.

19 The discussion then focused on the compliance of AR3 with Art. 56 EPC. The ED referred to the objection raised in the summons, which also applied to this request. The A put forward their arguments using the problem-solution approach. D1 was considered the closest prior art. In the A's view, the differences were the correct structure of eculizumab and the formulation defined in claim 1 (unit dosage containing 300 mg, as 30 ml of a 10 mg/ml solution). The effect derived from the difference was the particularly good stability to degradation shown in D32 (figs. 1, 2), D33 and D34 (figures). D33 showed that the aggregation of the antibody resulted in a reduction in the efficiency of the treatment (D33, item 8). Moreover, none of the cited documents pointed to the claimed concentration. In particular, D12 (par. [0074], [0171]) was not leading to the claimed solution, as it concerns nebulized forms, not injected forms. The

latter are the ones needed for the treatment of the claimed disease, namely paroxysmal nocturnal hemoglobinuria (PNH).

20 The proceedings were interrupted between 15:35 and 16:20. The ED announced that the AR3 did not comply with Art. 56 EPC. Moreover, the ED pointed out that this objection appeared to apply also to AR4-AR6.

21 After a short break requested by the A between 16:22 and 16:25, they requested to discuss **AR4**.

22 The A mentioned p. 19, l. 6-7 and p. 44 as the basis for the amendments. The meaning of the newly introduced feature "...is obtainable by transfecting cells with..." was discussed.

23 After a break between 16:40 and 16:50, the ED raised a further objection under 84 EPC. The interpretation of the claim was further discussed with proposals from both the ED and the A. One of the proposal of the A was in written form (annex E). None of these proposals were preliminarily considered by the ED to overcome the objection under Art. 84 EPC and none of them was formally presented at this point as a further request.

24 The ED then asked the A if they had further arguments concerning AR4. As they denied, the ED announced that AR4 was considered not to fulfil the requirements of Art. 123(2), 84 and 56 EPC. The ED mentioned that this objection also appeared to apply to AR6.

25 The applicant pointed out that **AR5** and **AR6** further contained the feature that the pharmaceutical composition was to be administered by intravenous infusion. In their view, this rendered the subject-matter inventive over D1 in combination with D12, as D12 only referred to a composition for nebulization. The ED pointed out that the intravenous administration was known from D1-D5. After short consultation with the members of the ED, the chairman announced that AR5 contravened Art. 56 EPC and AR6 did not comply with Art. 123(2), 84 and 56 EPC.

26 The chairman of the ED asked the A whether they had further requests, which was answered in the negative.

27 The proceedings were then interrupted between 17:10 and 17:30 to give the A time to provide the ED with AR3-6 in typed form (annexes F-I).

28 After noting that the new AR4 and AR6 differed from the handwritten form that had been previously discussed during the proceedings (see annexes G/I vs annexes B/D, respectively), the ED requested these versions be corrected. The A admitted that AR4-6 did not corresponded to the handwritten versions but

emphasized that it was their understanding that the versions of AR4 and AR6 as shown in annexes G and I had been discussed. The proceedings were interrupted between 17:33-17:55.

29 The A provided the corrected AR4 and AR6 (annexes J and K, respectively) corresponding to the handwritten requests (see annexes B and D, respectively). Moreover, the A requested to introduce two further requests into the proceedings (named AR4a and AR6a, respectively; see annexes L and M).

30 The admissibility of the new requests **AR4a** and **AR6a** was discussed. The ED asked for the reasons of the late filing and for the features in these requests that should overcome the objections raised for the previous requests, in particular Art. 56 EPC.

31 After a break between 18:05 and 18:20 the ED announced that AR4a and AR6a were not admitted into the proceedings.

32 The application was refused because the MR and AR1-2 did not comply with R. 139, Art. 76(1) and 123(2) EPC; AR3 and AR5 did not comply with Art. 56 EPC; and AR4 and AR6 did not comply with the requirements of Art. 123(2), 84 and 56 EPC; being AR3-6 as shown in annexes F, J, H and K, respectively.

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THIRD AUXILIARY

Annex A

~~MAIN~~ REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10 wherein the antibody is obtainable by production in transfected CHO or NSO cells.

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FOURTH AUXILIARY

~~MAIN~~ REQUEST

Annex B

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the transfected cells are CHO or NS0 cells.

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Annex D

SIXTH ~~SECOND~~ AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 10 wherein the transfected cells are CHO or NS0 cells.

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<comprises a heavy chain and a light chain and is obtainable by producing in transfected cells> Annex E

FIFTH

~~SECOND~~ AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises a heavy chain consis~~ting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use acording to any one of cleims 1 to 11 wherein the antibody is obtainable by production in transfected CHO or NSO cells.

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Annex C

FIFTH
~~SECOND~~ AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10 wherein the antibody is obtainable by production in transfected CHO or NSO cells.

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Annex F

THIRD AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

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~~MAIN~~ THIRD AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting ~~of residues 23 to 214~~ of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

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FOURTH AUXILIARY REQUEST
CLAIMS

Annex G

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the transfected cells are CHO or NS0 cells.

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~~THIRD~~ FOURTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV

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infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the ~~antibody is obtainable to production in~~ transfected cells are CHO or NS0 cells.

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Annex H

FIFTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

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~~SECOND~~ FIFTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

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Annex I

SIXTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the transfected cells are CHO or NS0 cells.

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~~FIFTH~~ SIXTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the ~~antibody is obtainable by production in~~ transfected cells are CHO or NS0 cells.

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Annex J

FOURTH AUXILIARY REQUEST
CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the transfected cells are CHO or NS0 cells.

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~~THIRD~~ FOURTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV

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infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the ~~antibody is obtainable to production in~~ transfected cells are CHO or NS0 cells.

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Annex K

SIXTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the transfected cells are CHO or NS0 cells.

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~~FIFTH~~ SIXTH AUXILIARY REQUEST

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody ~~comprises~~ is obtainable by transfecting cells with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the ~~antibody is obtainable by production in~~ transfected cells are CHO or NS0 cells.

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Annex L

AUXILIARY REQUEST 4A

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the transfected cells are CHO or NS0 cells.

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~~FOURTH~~ AUXILIARY REQUEST 4A

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected~~transfecting~~ cells ~~with~~ a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH).

2. The pharmaceutical composition for use according to claim 1, wherein the pharmaceutical composition is to be administered by intravenous infusion.

3. The pharmaceutical composition for use according to claim 2, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

5. The pharmaceutical composition for use according to any one of claims 1 to 4, which has a pH of 5 to 7.

6. The pharmaceutical composition for use according to claim 5, which has a pH of 7.

7. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the patient is anemic.

9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV

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infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

12. The pharmaceutical composition for use according to any one of claims 1 to 11, wherein the transfected cells are CHO or NS0 cells.

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Annex M

AUXILIARY REQUEST 6A

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the transfected cells are CHO or NS0 cells.

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~~SIXTH~~ AUXILIARY REQUEST 6A

CLAIMS

1. A pharmaceutical composition comprising an antibody that binds C5 in a 300mg single unit dosage form comprising 30 ml of a 10 mg/ml sterile, preservative free solution, wherein the antibody is obtainable by producing in transfected ~~transfecting~~ cells ~~with~~ a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the pharmaceutical composition is to be administered by intravenous infusion.

2. The pharmaceutical composition for use according to claim 1, wherein the antibody is diluted to a final concentration of 5 mg/ml prior to administration.

3. The pharmaceutical composition for use according to claim 1 or 2, wherein the patient is human and the antibody is to be administered at a dosage level of between 5 mg per kg and 50 mg per kg per treatment.

4. The pharmaceutical composition for use according to any one of claims 1 to 3, which has a pH of 5 to 7.

5. The pharmaceutical composition for use according to claim 4, which has a pH of 7.

6. The pharmaceutical composition for use according to any one of claims 1 to 5, which comprises an emulsifier.

7. The pharmaceutical composition for use according to any one of claims 1 to 6, wherein the patient is anemic.

8. The pharmaceutical composition for use according to any one of claims 1 to 7, wherein the antibody is administered as follows: 600 mg via 25 to 45 minutes IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose every 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter.

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9. The pharmaceutical composition for use according to any one of claims 1 to 8, wherein the impact of terminal complement inhibition by the antibody on chronic intravascular hemolysis in the PNH patient results in an immediate (one week) and sustained decrease in mean levels of LDH.

10. The pharmaceutical composition for use according to any one of claims 1 to 9, wherein administration of the antibody prolongs the health adjusted life expectancy of the patient suffering from PNH.

11. The pharmaceutical composition for use according to any one of claims 1 to 10, wherein the transfected cells are CHO or NS0 cells.

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Article 106
Decisions subject to appeal

(1) An appeal shall lie from decisions of the Receiving Section, Examining Divisions, Opposition Divisions and the Legal Division. It shall have suspensive effect.

(2) A decision which does not terminate proceedings as regards one of the parties can only be appealed together with the final decision, unless the decision allows a separate appeal.

(3) The right to file an appeal against decisions relating to the apportionment or fixing of costs in opposition proceedings may be restricted in the Implementing Regulations.

Rule 97
Appeal against apportionment and fixing of costs

(1) The apportionment of costs of opposition proceedings cannot be the sole subject of an appeal.

(2) A decision fixing the amount of costs of opposition proceedings cannot be appealed unless the amount exceeds that of the fee for appeal.

Rule 98
Surrender or lapse of the patent

The decision of an Opposition Division may be appealed even if the European patent has been surrendered in all the designated Contracting States or has lapsed in all those States.

Article 107
Persons entitled to appeal and to be parties to appeal proceedings

Any party to proceedings adversely affected by a decision may appeal. Any other parties to the proceedings shall be parties to the appeal proceedings as of right.

Article 108
Time limit and form

Notice of appeal shall be filed, in accordance with the Implementing Regulations, at the European Patent Office within **two months** of notification of the decision. Notice of appeal shall not be deemed to have been filed until the fee for appeal has been paid. Within **four months** of notification of the decision, a statement setting out the grounds of appeal shall be filed in accordance with the Implementing Regulations.

Further information concerning the filing of an appeal

(a) Notice of appeal can be filed in accordance with Rule 1 and Rule 2(1) EPC, by delivery by hand, by post, or by technical means of communication. The filing has to comply with the details and conditions and, where appropriate, any special formal or technical requirements laid down by the President of the European Patent Office (R. 99(3) EPC).

(b) The addresses of the filing offices of the European Patent Office are as follows:

(i) European Patent Office
D-80298 Munich
Germany

Fax: +49 89 2399-4465

(ii) European Patent Office
Postbus 5818
NL-2280 HV Rijswijk (ZH)
The Netherlands

Fax: +31 70 340-3016

(iii) European Patent Office
D-10958 Berlin
Germany

Fax: +49 30 259 01-840

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(c) The notice of appeal must contain the name and address of the appellant in accordance with the provisions of Rule 41(2)(c) EPC, an indication of the decision impugned, and a request defining the subject of the appeal. In the statement of grounds of appeal the appellant shall indicate the reasons for setting aside the decision impugned, or the extent to which it is to be amended, and the facts and evidence on which the appeal is based (R. 99(1) and (2) EPC). The notice of appeal and any subsequent submissions stating the grounds for appeal must be signed (R. 50(3) EPC).

(d) The fee for appeal is laid down in the Rules relating to Fees. The schedule of fees and expenses of the EPO or a reference to the current version is regularly published in the Official Journal of the European Patent Office under the heading "Guidance for the payment of fees, expenses and prices". Fee information is also published on the EPO website under www.epo.org/ fees.

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I. Summary of Facts and Submissions

1. European patent application No. 16 195 355.9 having the title "TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT" is a divisional application of EP11001632 and was filed on 15.03.2007. It claims priority of US 783070 filed on 15.03.2006. The applicant is

Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510
US.

2. The following documents were cited in the course of the procedure:

D1 US 2005/191298

D2 Hillmen et al. (2003)

D3 Hillmen et al. (2004)

D4 Harding (2004)

D5 Hill (2005)

D6 CAS database registry

D7 Thomas et al. (1996)

D8 Table of Alexion European patents and patent applications referring to "eculizumab"

D9 Submission of Alexion dated 30 December 2013 in opposition proceedings against EP 1720571

D10 WO 2005/074607

D11 US 6355245

D12 Bell declaration (filed 10 July 2017)

D13 Boone declaration (filed 10 July 2017)

D14 Andrien declaration (filed 10 July 2017)

D15 Letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007 (filed 16 January 2018)

D16 Press release made by Alexion Pharmaceuticals on 16 March 2007 (filed 16 January 2018)

D17 Saxe declaration (filed 16 January 2018)

D18 Experimental report (filed 16 January 2018)

D19 Second Andrien declaration (filed 15 August 2018)

D20 Extract from Alberts et al. (1994) (filed 15 August 2018)

D21 Opposition statement filed by Pfizer against EP 1720571 (filed 15 August 2018)

D22 Redacted version of clinical trial protocol C04-001 (filed 15 August 2018)

D23 Redacted version of clinical trial protocol C02-002 (filed 15 August 2018)

D24 Table of Alexion patents and patent applications showing how these reference D7 and D11, and providing the status of each case

D25 Summons and preliminary opinion of Opposition Division in opposition against EP 1720571

D26 Response filed by Alexion on 17 March 2014 to summons and preliminary opinion in opposition against EP 1720571

D27 Decision revoking EP 1720571

D28 Annotated version of original CAS Registry No. 219685-50-4 obtained from http://commonchemistry.org/ showing the errors in the sequences

D28a Annotated version of corrected CAS Registry No. 219685-50-4 obtained from CAS

D29 Second Experimental Report

D30 Wang et al. (2007) J. Pharmaceutical Sciences 96(1): 1-26

D31 Bombara declaration

3. In the EESR, D1-D6 were cited and objections under Articles 76(1), 54 and 56 EPC against the subject-matter of the claims were raised.

4. In the response dated 10.07.2017, the applicant provided its arguments to the raised objections and requested the "Correction of an obvious error under Rule 139 EPC". In particular, the applicant argued that

(a) claim 1 found a clear and unambiguous basis in the parent application as filed on page 9, last para. in combination with pages 44 and 45. The skilled person only needed to combine features of a broader embodiment and a narrower embodiment both characterising the structure of eculizumab to arrive at the subject-matter of claim 1. This constituted an allowable intermediate generalisation since none of the features were inextricably linked to other features.

(b) the biochemical structure of eculizumab was not publicly available before the priority date of the present application. Eculizumab was not disclosed in the prior art or provided to a member of the public without strict confidentiality and storage restrictions; especially not the unique, non-naturally occurring, protein-engineered full heavy chain of SEQ ID NO:2, as indicated in the declarations of Dr. Bell (D12) and Dr. Boone (D13). D1-D5 referred to confidential clinical trials. Their authors were bound to confidentiality and the documents did not disclose the structure of eculizumab.

(c) it was obvious to the skilled person that 22 amino acids needed to be deleted from the sequences 3 and 4. They corresponded to the leading peptide of the sequence of the light chain of the claimed antibody (i.e. eculizumab), which could not be present in the mature form of the antibody. In support of this request the applicant annexed the declaration of Dr. Bruce Andrien (D14).

5. In a communication under Article 94(3) EPC issued by the examining division on 17.11.2017 the objections raised in the ESOP under Articles 76(1), 54 and 56 EPC were maintained. Moreover, the examining division raised objections under Articles 123(2) and 83 EPC in relation to the correction under Article 139 EPC requested by the applicant.

Document D7 was annexed to the communication from the EPO.

6. On 12.12.2017, Third Party Observations were received. These pointed out the following:

(a) in the course of the procedure for previous patents and patent application the applicant had stated that the structure of eculizumab was known in the art (in particular, reference was made to the opposition proceedings for EP17201571 - see D8 and D9- and to D6);

(b) by stating the opposite in the present proceedings, the applicant contravened against the principle of *nemo potest venire contra factum proprium*;

(c) the subject-matter was not novel over the disclosure of D1-D5.

7. In the response received on 16.01.2018 the applicant requested further examination on an amended set of claims 1-2 and amended description page 5 and provided arguments in support of the patentability of these claims.

In particular, (a) the description was amended in order to correct the allegedly obvious error under Article 139 EPC, (b) it was pointed out that the structure of eculizumab was not available to the public at the filing date of the present application since the sequence of eculizumab provided to CAS for publication in 1999 was inadvertently incorrect. The mistake was not corrected until May 2009, which is after the filing date of the present application. Marketing authorisation for eculizumab was not obtained until 16 March 2007; therefore eculizumab was not available to the market before that date. The Third Party Observations were argued to be merely circumstantial.

Documents D15-D18 were annexed to the letter in support of the arguments of the applicant.

8. On 7 May 2018 the examining division summoned to oral proceedings on 5 February 2019. The division maintained its opinion with regard to the inadmissibility of the correction and concluded that the requirements of Articles 76(1) and 123(2) EPC were not met. It also pointed out that it was of the opinion that the structure of eculizumab was available to the public at least as of February 1999, and that the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. The division thus concluded that D1 – D5 and D10 were novelty destroying and that the requests on file lacked inventive step. The division also pointed out that – had the structure of eculizumab at the date of filing of the present application only been disclosed in the incorrect form submitted to the CAS registry – the skilled person would not have known which structure was to be assigned to eculizumab and the application was not sufficiently disclosed under Article 83 EPC.

9. In a response to the summons the applicant submitted D19-D23 on 15.08.2018, and provided arguments in support of the patentability of the claims.

10. On 2 January 2019 the applicant was informed that the oral proceedings were cancelled because the division was to be enlarged by a legally qualified member and that a new date will be set.

11. On 27.03.2019 the examining division issued a new summons to oral proceedings to be held on 05.09.2019. The examining division informed the applicant of the fact that the examining division had been enlarged under Article 18(2) EPC by a legally qualified member and that the proceedings were combined with the proceedings of the parallel case EP16 172 335.8 for the purpose of the oral proceedings for reasons of procedural economy. The objections and arguments previously raised were confirmed.

D10 and D11 were introduced in the procedure.

12. In a response to the summons dated 05.07.2019 the applicant submitted auxiliary requests 1-3 and documents D24 to D31 and provided further arguments in support of the patentability of the claims.

13. On 02.09.2019, Third Party Observations were received underlying the fact that the applicant acted against the principle of *nemo potest venire contra factum proprium*.

On 04.09.2019, Third Party Observations were received objecting against the subject-matter of the claims under Articles 76(1) and 123(2) EPC.

14. On 05.09.2019, oral proceedings were held. In the course of the proceedings, the applicant's representatives was given time to consider the Third Party Observations dated 04.09.2019 which they were not aware of. After the discussion of admissibility of correction under Rule 139 EPC and extension of subject-matter under Article 123(2) and 76 EPC the main and all auxiliary requests were rejected. The discussion of the substantial matter was nevertheless continued in order to obviate a possible remittal under Article 111 EPC and to allow the applicant the possibility to file a further request that overcame all objections which had been expressed in the various communications and especially in the summons to oral proceedings. After the discussion of novelty, the applicant requested an adjournment of the oral proceedings in order to seek legal advice. The adjournment was not granted by the examining division. The patent application was refused after no further requests were filed.

15. The decision to refuse the present application is based on the following requests:

Main Request

Description, Pages

1-4, 6-55	as originally filed	
5	filed in electronic form on	16-01-2018

Sequence listings, SEQ ID NO

1-10	as originally filed	

Claims, Numbers

1, 2	filed in electronic form on	16-01-2018

Drawings, Sheets

1/4-4/4	as originally filed	

Auxiliary Request 1

Description, Pages

1-4, 6-55	as originally filed	
5	filed in electronic form on	16-01-2018

Sequence listings, SEQ ID NO

1-10	as originally filed	

Claims, Numbers

1, 2	filed in electronic form on	05-07-2019

Drawings, Sheets

1/4-4/4	as originally filed	

Auxiliary Request 2

Description, Pages

1-4, 6-55	as originally filed	
5	filed in electronic form on	16-01-2018

Sequence listings, SEQ ID NO

1-10	as originally filed	

Claims, Numbers

1, 2	filed in electronic form on	05-07-2019

Drawings, Sheets

1/4-4/4	as originally filed	

Auxiliary Request 3

Description, Pages

1-4, 6-55	as originally filed	
5	filed in electronic form on	16-01-2018

Sequence listings, SEQ ID NO

1-10	as originally filed	

Claims, Numbers

1, 2	filed in electronic form on	05-07-2019

Drawings, Sheets

1/4-4/4	as originally filed	

II. Reasons for the Decision

1) Main request

The claims of the main request read as follows:

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of residues 23-214 of SEQ ID NO:4.

2. A pharmaceutical composition comprising the antibody of claim 1.

1.1 Articles 76(1) and 123(2) EPC

The present application does not meet the requirements of Articles 123(2) and 76(1) EPC for the following reasons.

1.1.1 With the amendments filed on 16.01.2018, the applicant corrected page 5 of the description of the application (which is the same as the description of the 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.

Although the SEQ ID NO: 3 and SEQ ID NO:4 had already been amended on 10.07.2017 (on page 44 of the application) and nowhere in the course of the procedure has it been stated that this amendment was withdrawn, it is clear to the examining division that the amendments carried out on 16.01.19 on page 5 of the application and on claim 1 of the main request relate to the sequences as originally filed.

In particular, instead of the full length of SEQ ID NO:3 and SEQ ID NO: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 original 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 original SEQ ID NO:4.

In his letter dated 16.01.2018, the applicant explained that the amendments carried out were to be considered the correction of an obvious mistake under Rule 139 EPC.

The applicant provided its arguments on this point also in its letters dated 15.08.2018 and 05.07.19 and at the oral proceedings held on 05.09.19. The applicant supported its arguments by referring to D14, D19 and D20.

The argument of the applicant can be summarised as follows:

(I) The originally claimed sequences erroneously comprise the N-terminal 22-amino acids-long leader peptide which however cannot be present in the mature light chain comprised in the mature antibody. The corrected sequences therefore represent the sequences of the light chain of eculizumab in its mature form.

(II) The skilled person would recognise that SEQ ID NO:3 and SEQ ID NO:4 are not correct, i.e. comprise a leader peptide, because (i) they start with a methionine, and in particular with the amino acids MDM, and residues 21 and 22 are Arg and Cys respectively, (ii) the methionine is followed by 21 amino acids which are mostly hydrophobic, (iii) SEQ ID NO:4 with 236 amino acids is longer than a typical mature antibody light chain, and (iv) the mature form of kappa antibody light chains usually

starts with the amino acids DIQM, i.e. Asp-Ile-Gln-Met (see sections 8-12 of D14 and in particular the Kabat document which is an abstract of a longer documents providing examples of kappa antibodies - Exhibit E of D14).

Moreover, antibodies are produced using cell-based recombinant methods (see D19) which result in the N-terminal leader peptide being cleaved off such that it is not present in the mature form of the antibody.

(III) The correction carried out by the applicant is derivable by the skilled person directly and unambiguously from the application as filed, using common general knowledge. Therefore the skilled person would have recognised immediately that the applicant did not have the intention to seek protection for an antibody that contains a leader peptide.

(IV) The EPO accepted corrections of this type in EP1558648 and EP2581113.

1.1.2 The examining division carefully considered the arguments of the applicant and notes the following:

a) Nowhere in the application is it indicated that the sequence for which protection is sought has to be in its mature form, or that a leader peptide has to be cleaved off from the immature form of the light chain represented by originally filed SEQ ID NO:3 or SEQ ID NO:4.

b) It is not contested that there may be usual starting and/or ending amino acid(s) for a leading peptide and for the mature form of the light chain of an antibody as indicated by the applicant. However, this does not mean that these usual amino acids or sequences of amino acids are present in the totality of the light chains of antibodies. Therefore, the skilled person may not have derived immediately the presence of a leading peptide in SEQ ID NO:3 and SEQ ID NO:4.

Moreover, even if the skilled person could have thought that SEQ ID NO:3 and SEQ ID NO:4 included a leading peptide (which is not conceded), it wouldn't have been possible to identify the exact length of the leading peptide without a certain level of doubt. Indeed the documents referred to by the applicant refer to usual or common features of a leading peptide or a mature antibody sequence, but do not exclude completely the possibility of deviations from these features. Especially in the patent field, deviation can mean innovation.

c) 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. This is particularly true in the present case since the application relates to the field of engineered antibodies and not of naturally occurring human antibodies (such as those mentioned in the Kabat document mentioned in D14).

d) It cannot be excluded with certainty that after production of the mature antibody in the transfected cells (which is only one possible way of producing the claimed antibody - see page 19 of the application), a sequence of amino acids is attached to the N-terminal amino acid.

e) Every patent application has to be assessed individually on the basis of its own merits. The examining division responsible for the present case is not bound by any decisions taken by other examining divisions with regard to other applications and on the basis of different merits.

1.1.3 Rule 139 EPC stipulates that mistakes in any document filed with the European Patent Office may be corrected on request if the correction is obvious in the sense that it is immediately evident that nothing else would have been intended than what is offered as the correction. For the reasons given above, however, the examining division cannot exclude that the correction carried out by the applicant is obvious and concludes that the amendments to the present application cannot be considered as the correction of an obvious error under Rule 139 EPC.

For this reason the subject-matter of the present application is not considered to meet the requirements of Articles 76(1) and 123(2) EPC.

2) Auxiliary request 1

The claims of the auxiliary request 1 read as follows:

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO:2 and the light chain amino acid sequence shown in SEQ ID NO:4.

2. A pharmaceutical composition comprising the antibody of claim 1.

2.1 Articles 76(1) and 123(2)EPC

The reasoning applied to the assessment of whether the present application meets the requirements of Articles 76(1) and 123(2) EPC exposed in item 1 above and the conclusions reached therein, apply also to the subject-matter of the auxiliary request 1.

At the oral proceedings, the applicant indicated page 4, line 32 in combination with page 44 of the application as filed as basis for the subject-matter of amended claim 1 of the auxiliary request 1.

The examining division investigated the reasons for choosing a wording that was not exactly the one used in the application as filed for defining the claimed antibody.

The applicant explained that the wording "shown in" used in claim 1 of the auxiliary request 1 was chosen in order to correct the mistake made by the applicant when filing SEQ ID NO:3 and SEQ ID NO:4, i.e. including the leading peptide in the sequences. Therefore, the wording used in the claims intended to cover antibodies comprising parts of SEQ ID NO:2 and SEQ ID NO:4 and in particular antibodies in which the light chain did not include the leading peptide.

The examining division considered the arguments of the applicant and came to the following conclusions.

a) The expression "shown in" used in claim 1 of the auxiliary request 1 is not disclosed as such in the original/parent application in relation to the claimed antibody.

b) The interpretation given by the applicant to that expression clearly implies that the subject-matter of the claims of auxiliary request 1 extends in scope well beyond the subject-matter of the claims of the main request, which is already considered not to comply with the requirements of Articles 123(2) and 76(1) EPC by the examining division (see item 1 above).

It follows from the considerations under items 3.1 and 3.2 above, that the subject-matter of the auxiliary request 1 contravenes the requirements of both Articles 123(2) and 76(1) EPC.

3) Auxiliary request 2

The claims of auxiliary request 2 read as follows:

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4 for use in treating paroxysmal nocturnal hemoglobinuria.

3.1 Articles 76(1) and 123(2)EPC

The reasoning applied to the assessment of whether the present application meets the requirements of Articles 76(1) and 123(2) EPC exposed in items 1 and 3 above and the conclusions reached therein, apply also to the subject-matter of the auxiliary request 2.

It follows from the considerations under item 1.1 above, that the subject-matter of the auxiliary request 2 contravenes the requirements of both Articles 123(2) and 76(1) EPC.

4) Auxiliary request 3

The claims of auxiliary request 3 read as follows:

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO:2 and the light chain amino acid sequence shown in SEQ ID NO:4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO:2 and the light chain amino acid sequence shown in SEQ ID NO:4 for use in treating paroxysmal nocturnal hemoglobinuria.

4.1 Articles 76(1) and 123(2)EPC

The reasoning applied to the assessment of whether the present application meets the requirements of Articles 76(1) and 123(2) EPC exposed in items 1 and 3 above and the conclusions reached therein, apply also to the subject-matter of auxiliary request 3.

It follows that the subject-matter of the auxiliary request 3 contravenes the requirements of both Articles 123(2) and 76(1) EPC.

5) Additional reasons

5.1 Article 54 EPC

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of none of the claims of the main or the auxiliary requests is new within the meaning of Article 54(1) and (2) EPC.

5.1.1 Eculizumab is considered to have been available to the public before the filing date of the present application

The applicant argued that even though the structure of eculizumab had been submitted to CAS in February 1999 it was not disclosed to the public since it had inadvertently been submitted with an error. The correction of this error was not obvious. A product containing eculizumab was not available on the market before marketing authorisation for eculizumab was granted to the applicant by the FDA in March 2007. In the meantime all persons with access to eculizumab were bound by confidentiality agreements.

However, the examining division is of the opinion that confidentiality agreements cannot relate to information that is (believed to be) in the public domain. The scientists working with eculizumab were under the impression that the structure of eculizumab had been

submitted to CAS. Hence, they were not bound to confidentiality with regard to the structure of eculizumab and could have performed a sequencing of the antibody with the general knowledge available in the art without technical or legal constraints. Whether or not any one of them indeed performed such a sequencing is irrelevant for the question of the availability of the structure of eculizumab to the public. The examining division thus concludes that the structure of eculizumab was at least available to the scientists working with eculizumab without these scientist being bound to confidentiality irrespective of whether the structure submitted to CAS in February 1999 was erroneous or not. This line of argument is supported by the wording of the confidentiality agreements cited by the applicant in its brief of 10 July 2017 (declaration of Dr. Laural Boone, sec. 7 and 8) and submitted as annex to its brief of 15 August 2018 (Study protocol, sec. 11.1) and of 5 July 2019 (Master Clinical Study Agreement, sec. 7). In all instances (alleged) signatories to the agreements are bound to confidentiality only with regard to unpublished information or information that has not entered the public domain.

The applicant submitted during the oral proceedings that this conclusion of the examining division had not been mentioned in the division's preliminary opinion. The applicant contended that it was not sufficient to be given the possibility to be heard on this issue during the oral proceedings because it was a legal issue that necessitated obtaining a legal opinion with regard to the extent of the wording of the confidentiality agreement. The applicant therefore requested adjournment of the proceedings. The examining division did not grant the request for the following reasons:

a. In all of its communications during the examining procedure the examining division had pointed to the fact that it regarded eculizumab to have been available to the public before the priority date of the present application and that any scientist working with eculizumab could have performed a sequencing (e.g. communication of 17.11.2017, page 3; summons of 07.05.2018, page 2). This opinion was maintained also after the applicant submitted (references to) the respective confidentiality agreements (summons of 27.03.2019, page 4). It could not have come as a surprise to the applicant that the division continued to maintain its position. Indeed, the argument of the examining division was known, the only new information being the reference to the specific passage in the Chemical Abstract, merely confirming the examining division's position.

b. Even if the examining division's conclusion was considered to be new, it was an argument or a legal consideration based on facts already introduced into the proceedings. Such arguments can arise any time during oral proceedings. In addition, during the oral proceedings the applicant was explicitly asked why the signatories of the confidentiality agreements/clauses should have considered the structure of eculizumab not to be within the public domain even though the structure had been submitted to CAS and even the applicant was unaware of the alleged mistake. Therefore, the examining

division considers the applicant to have had sufficient opportunity to be heard on the issue. After all, parties are expected to prepare themselves fully for oral proceedings (Guidelines, Part E-III, 8.11.1).

c. The question of novelty was only discussed as "additional reason" after the main and all auxiliary requests were held not to meet the requirements of the EPC. Even though the examining division gave the applicant ample opportunity to formulate a new request also taking further possible objections into account, the applicant chose not to file any new requests. However, it is standing case law of the boards of appeal that "additional reasons" are provided as voluntary information to an applicant on the preliminary opinion of the examining division. They can thus not violate a party's right to be heard (see T 726/10 and T 725/05). Neither can they justify the adjournment of the proceedings.

5.1.2 The main request of the present application also lacks novelty in view of the disclosure of D1-D5

Since the correct structure of eculizumab has been considered to be available to the public at the filing date of the present application, 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.

5.1.3 The main request of 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.

5.1.4 The main request of the present application also lacks novelty over the disclosure of D11

The description of the invention (see page 16, lines 19- 24) refers to D11 and states that the document discloses the preferred whole antibody named eculizumab. That would mean that the eculizumab claimed in the patent and the one disclosed in D11 is the same antibody.

5.1.5 Auxiliary Request 1

The subject-matter of auxiliary request 1 does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1 and 2 is not new within the meaning of Article 54(1) and (2) EPC for the same reasons given above.

5.1.6 Auxiliary Request 2

The subject-matter of auxiliary request 2 does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1 and 2 is not new within the meaning of Article 54(1) and (2) EPC for the same reasons given above.

As a matter of fact, D1-D5 and D10 all disclose the eculizumab for use in the treatment of paroxysmal nocturnal hemoglobinuria.

5.1.7 Auxiliary Request 3

The subject-matter of auxiliary request 3 does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1 and 2 is not new within the meaning of Article 54(1) and (2) EPC for the same reasons given above.

6) Decision

The examining division is of the opinion that the present European patent application does not meet the requirements of the European Patent Convention and therefore refuses the application under Article 97(2) EPC.

Samsung Bioepis Exhibit No. 1028
Page 1447

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Application No.	Ref.	Date
16 195 355.9 - 1112		11.02.2020
Applicant Alexion Pharmaceuticals, Inc.		

Decision to refuse a European Patent application

The Examining Division - at the oral proceedings dated 05.09.2019 - has decided:

European Patent application No. 16 195 355.9 is refused.

Applicant/s:
Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510
US

Title
TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

The grounds for the decision are set out on the supplemental sheets annexed hereto.

Means of redress

This decision is open to appeal.
Attention is drawn to the attached text of Articles 106 to 108 EPC and Rules 97 and 98 EPC.

Registered letter
EPO Form 2007 07.19TRI

Examining Division:

Chairman:	Orlando, Michele
2nd Examiner:	Rodríguez-Palmero, M
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Enclosure(s): 15 page/s reasons (Form 2916)
Form 2019

to EPO postal service: 06.02.20

Registered letter
EPO Form 2007 07.19TRI

J A • KEMP

BY ONLINE FILING

The European Patent Office
80298 Munich
Germany

24 March 2020

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

We give notice of appeal against the examining division's decision of 11 February 2020 to refuse this application.

Please debit the appeal fee (2255 EUR) from J A Kemp LLP's deposit account 2805.0038. We request that any underpayment of this fee be deducted from, or any overpayment of this fee be credited to, deposit account 2805.0038.

The name and address of the appellant are as follows:

Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510 / US

The impugned decision is the decision of the examining division of 11 February 2020.

The appellant requests that the decision be set aside and that the application be allowed on the basis of the main request or, failing that, on the basis of the auxiliary requests on file.

We request oral proceedings in the event that the board is minded not to allow the main request.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
FOR J A KEMP LLP
ASSOCIATION NO. 811

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European Patent Office
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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Notice of appeal	File Notice of Appeal N411599EP-B PXT.PDF	APPEAL.PDF

	Fees	Factor applied	Fee schedule	Amount to be paid
15-1	011 Appeal fee for an appeal filed by an entity other than those referred to in Rule 6(4) and (5) EPC	1	2 255.00	2 255.00
	Total:		**EUR**	**2 255.00**

	Payment	
1	Method of payment	Debit from deposit account
	Currency:	EUR
	The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated on the fees page.	
	Deposit account number:	28050038
	Account holder:	J A Kemp LLP
2	Refund/Reimburserment	
	Reimbursement (if any) to be made to EPO deposit account:	28050038
	Account holder:	J A Kemp LLP

Signatures

Place:	**Oxford, GB**
Date:	**24 March 2020**
Signed by:	**Pamela Tuxworth 23470**
Association:	**J A Kemp LLP**

Representative name:	**TUXWORTH, Pamela Mary**
Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1028
Page 1452

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	8464756	
Application number	EP16195355.9	
Date of receipt	24 March 2020	
Receiving Office	European Patent Office, The Hague	
Your reference	N411599EP-B	
Applicant	All applicants as on file	
Documents submitted	package-data.xml epf1038.pdf (2 p.)	ep-sfd-request.xml APPEAL.PDF\File Notice of Appeal N411599EP-B PXT.PDF (1 p.)
Submitted by	CN=Pamela Tuxworth 23470	
Method of submission	Online	
Date and time receipt generated	24 March 2020, 16:15 (CET)	
Message Digest	0D:47:8D:A5:00:6A:F9:D6:95:20:A8:40:70:93:BE:F9:08:19:5C:F2	

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/

J A · K E M P

19 June 2020

Our Ref: N411599EP-B

STATEMENT OF GROUNDS OF APPEAL

Application No: 16195355.9

Applicant: Alexion Pharmaceuticals, Inc.

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CONTENTS

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3 CITED DOCUMENTS 3

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7 INVENTIVE STEP 27

1 Requests

1.1 We request that the decision of the examining division dated 11 February 2020 be set aside and that the application be allowed on the basis of the Main Request or, failing that, on the basis of one of Auxiliary Requests 1 to 11. Comments on the Auxiliary Requests are set out below.

1.2 We request oral proceedings in the event that the board is minded not to allow the Main Request.

2 Main and Auxiliary Requests

2.1 The Main Request is identical to the claims filed on 16 January 2018.

2.2 Auxiliary Requests 1 to 4 are amendments which relate to the examining division's objection of added matter and can be summarised as follows:

- Claim 1 of Auxiliary Request 1 specifies that the antibody is eculizumab and defines the heavy chain as consisting of SEQ ID NO: 2. The light chain is not defined in this claim. Basis for the amendment can be found at page 4, line 32 which discloses that antibody is eculizumab as well as page 44, line 8 which discloses the heavy chain of eculizumab as SEQ ID NO: 2 (page 4, line 32 and page 44, line 8 of the parent application).

- Claim 1 of Auxiliary Request 2 specifies that the antibody is obtainable by producing in transfected cells the defined heavy and light chains. Claim 2 recites that the transfected cells are CHO or NS0 cells. Basis for these amendments can be found in the application as filed at page 19, lines 6-7 (page 19, lines 6-7 of the parent application).

- Claim 1 of Auxiliary Request 3 specifies that the antibody is obtainable by producing in transfected CHO or NS0 cells the defined heavy and light chains. Basis for the amendment can be found in the application as filed at page 19, lines 6-7 (page 19, lines 6-7 of the parent application).

- Auxiliary Request 4 corresponds to Auxiliary Request 1 considered by the examining division. Claim 1 of Auxiliary Request 4 specifies that the heavy chain sequence is shown in SEQ ID NO: 2 and that the light chain sequence is shown in SEQ ID NO: 4. This amendment finds basis at page 4, line 32 and page 44 of the application as filed - page 4 discloses that the antibody is eculizumab and the heavy and light chain amino acid sequences of eculizumab are shown in SEQ ID NO: 2 and SEQ ID NO: 4, respectively, at page 44 (page 4, line 32 and page 44 of the parent application).

- Claim 1 of Auxiliary Request 5 specifies that the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4. This claim finds basis at page 5, lines 30-33 of the application as filed (page 5, lines 30-33 of the parent application).

2.3 Auxiliary Requests 6 to 11 correspond to the Main Request and Auxiliary Requests 1 to 5, respectively, but the claims have been rewritten as purpose limited product claims reciting that the antibody is for use in treating paroxysmal nocturnal hemoglobinuria. Basis for this amendment may be found in the application as filed at, for example, page 3, lines 24-28 (page 3, lines 24-28 of the parent application). These requests correspond to those before the examining division as follows:

- Auxiliary Request 6 corresponds to Auxiliary Request 2 considered by the examining division;

- Auxiliary Request 7 is a new request corresponding to new Auxiliary Request 1 above, with the additional limitation that the antibody is for use in treating paroxysmal nocturnal hemoglobinuria;

- Auxiliary Request 8 is a new request corresponding to new Auxiliary Request 2 above, with the additional limitation that the antibody is for use in treating paroxysmal nocturnal hemoglobinuria;

- Auxiliary Request 9 is a new request corresponding to new Auxiliary Request 3 above, with the additional limitation that the antibody is for use in treating paroxysmal nocturnal hemoglobinuria;
- Auxiliary Request 10 corresponds to Auxiliary Request 3 considered by the examining division;
- Auxiliary Request 11 corresponds to new Auxiliary Request 5 above, with the additional limitation that the antibody is for use in treating paroxysmal nocturnal hemoglobinuria.

3 Cited Documents

3.1 We refer to all of the documents cited during prosecution of this application. We are also filing herewith:

D32: a third declaration of Dr Bruce Andrien, providing comments in response to arguments made for the first time by the examining division in the decision;

D33: the review article Laffly and Sodoyer, "Monoclonal and recombinant antibodies, 30 years after...", Human Antibodies (2005) 33-55 which is cited in the third declaration of Dr Bruce Andrien and provides evidence of common general knowledge;

D34: declaration of Mr Andrew Webb providing legal interpretation of the clinical trial study agreements; and

D35: declaration of Dr Anita Hill, who was involved in some of the clinical trials carried out at Leeds Teaching Hospitals in relation to eculizumab.

4 Annexes

Annex 1: Submission of 5 July 2019.

Annex 2: Submission of 15 August 2018.

Annex 3: Submission of 16 January 2018.

5 **No Added Matter**

5.1 *Correction allowable under Rule 139 EPC*

5.1.1 The correction made to the application to define the antibody that binds C5 as having a light chain consisting of residues 23-214 of SEQ ID NO: 4, and the corresponding amendments to the definition of an antibody comprising a light chain consisting of SEQ ID NO: 4 or a light chain variable region consisting of SEQ ID NO: 3 in the description, meet with the requirements of Rule 139 EPC. Contrary to the conclusion of the examining division, it is immediately evident that nothing else would have been intended other than what is offered as the correction.

5.1.2 As an initial point, when considering correction under Rule 139 EPC, it is necessary to view the application from the viewpoint of the skilled person in light of his common general knowledge, as established by the Enlarged Board of Appeal in G3/89 and G11/91 (Headnote 1):

> *"The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC"*

5.1.3 The application is concerned with treatment of paroxysmal nocturnal hemoglobinuria (PNH), and the claimed antibody is a therapeutic antibody. The skilled person is therefore familiar with the structure and other properties of therapeutic antibodies and with processes for producing therapeutic antibodies.

5.1.4 The skilled person would have known from his common general knowledge that antibodies are secreted proteins produced from precursor light and heavy chain polypeptides, which precursor polypeptides each comprise (i)

a signal peptide (also referred to as a leader peptide) that is cleaved off by a peptidase in the endoplasmic reticulum (ER) of a cell and (ii) a mature polypeptide. As explained in paragraphs 9 to 12 of D19 and evidenced by the prior art textbook Alberts et al. (D20), which explains the fate of the signal peptide in the context of proteins generally, the signal peptide remains in the ER membrane whilst the mature polypeptide folds in the lumen of the ER to form the mature protein.

5.1.5 The skilled person would know that recombinant chimeric and humanised antibodies are suited for use as therapeutic antibodies because they are more similar to naturally occurring human antibodies and so have reduced immunogenicity in humans compared to murine antibodies (see D30, a review relating to the structure, instability and formulation of therapeutic antibodies, section headed "Alternative Forms of Antibodies" spanning pages 7 and 8, and the first paragraph of page 8 in particular; Laffly & Sodoyer (D33), a prior art review of monoclonal and recombinant antibodies, page 34, left column, particularly, first paragraph lines 9-21, second paragraph, and third paragraph, lines 1-2; and the third declaration of Dr Andrien (D32), paragraph 20). Such recombinant therapeutic antibodies are produced in cells that express the antibody heavy and light chains from DNA vectors transfected into the cells (see D30, section headed "Oligosaccharides" at page 6; D33, page 45, right column, lines 1-4 and page 46, left column, last paragraph; and the third declaration of Dr Andrien (D32) paragraph 15). No non-cell-based methods of producing recombinant therapeutic antibodies were known in the art (see D32, paragraph 15).

5.1.6 Further, the present application explicitly discloses that monoclonal antibodies may be produced in transfected cells, such as CHO cells and NS0 cells (page 19, lines 6 to 7 of the application).

5.1.7 The skilled person would therefore know with certainty that an engineered therapeutic antibody comprising a heavy chain and a light chain is produced in cells and therefore that a signal peptide is never present in the therapeutic antibody.

5.1.8 The skilled person would also be able to identify whether a given light chain amino acid sequence is that of the precursor polypeptide containing the signal peptide or that of the mature polypeptide (not containing the signal peptide) on the basis of his common general knowledge. The skilled person would immediately recognise the signal peptide in SEQ ID NO: 3 and SEQ ID NO: 4 by the starting methionine residue, and the stretch of 21 mainly hydrophobic amino acids (see paragraph 8 of D14). It would therefore be immediately apparent to the skilled person that SEQ ID NO:3 and SEQ ID NO:4 both include the amino acids of the signal peptide and that defining the therapeutic antibody as comprising a light chain variable region consisting of SEQ ID NO:3 or a light chain consisting of SEQ ID NO:4 is an error.

5.1.9 Further, the skilled person would know exactly where in SEQ ID NO:3 and SEQ ID NO:4 the signal peptide ends and the mature polypeptide begins. As explained in paragraphs 9-12 of the first Andrien declaration (D14), the amino acid sequences of signal peptides of many antibody light chains were part of the common general knowledge of the skilled person. Further, as explained in the third Andrien declaration (D32) at paragraph 12, it can be seen from Exhibit D of D14 "SIGNAL PEPTIDE OF HUMAN KAPPA LIGHT CHAINS" that in every antibody light chain precursor polypeptide which contains the sequence Gly-Ala-Arg-Cys (GARC) at positions 19-22, these are the last four amino acids of the signal peptide (amino acids -4, -3, -2 and -1). Similarly, as can be seen from Exhibit E of D14 "HUMAN KAPPA LIGHT CHAINS SUBGROUP I", in all cases where the light chain precursor polypeptide contains the sequence Asp-Ile-Gln-Met (DIQM) at residues 23-26, these are the first four amino acids of the mature polypeptide (amino acids 1-4).

5.1.10 The amino acids at positions 19-22 of SEQ ID NO:3 and SEQ ID NO: 4 are Gly-Ala-Arg-Cys (GARC), and the amino acids at positions 23-26 of SEQ ID NO:3 and SEQ ID NO: 4 are Asp-Ile-Gln-Met (DIQM). Thus, there would have been no doubt in the mind of the skilled person that SEQ ID NO: 3 and SEQ ID NO: 4 in the application as filed contain a signal peptide at positions 1-22 and that the amino acid sequence of the mature antibody is

at residues 23-214 of SEQ ID NO: 4 or residues 23-131 of SEQ ID NO: 3 (paragraph 13 of D32; see also D14, paragraphs 9-12).

5.1.11 Therefore, it would be immediately evident to the skilled person that the applicant would have intended nothing other than to define the antibody as comprising a light chain variable region consisting of only the mature portion beginning at position 23 of SEQ ID NO: 3 or a light chain consisting of only the mature portion beginning at position 23 of SEQ ID NO: 4.

5.1.12 The specific points considered by the examining division in reaching its incorrect conclusion that the correction does not satisfy the requirements of Rule 139 EPC are addressed below.

5.2 *Therapeutic antibodies never contain signal peptides*

5.2.1 In paragraph 1.1.2 (a) of the decision, the examining division states that:

> *"Nowhere in the application is it indicated that the sequence for which protection is sought has to be in its mature form, or that a leader peptide has to be cleaved off from the immature form of the light chain represented by originally filed SEQ ID NO: 3 or SEQ ID NO: 4."*

5.2.2 The applicant is seeking protection for a therapeutic antibody defined functionally by its binding to C5, and structurally in terms of the amino acid sequences of which its heavy and light chains are composed.

5.2.3 It is not necessary for the application to explicitly indicate that the antibody contains a mature light chain in order for the skilled person to know that the applicant made a mistake in defining the structure of the light chain of the antibody as consisting of the whole (precursor) light chain polypeptide including the signal peptide (see the third Andrien declaration (D32), paragraph 9).

5.2.4 It is, as explained in paragraphs 5.1.3 to 5.1.7 above, part of the skilled person's common general knowledge that the light chain of a therapeutic

antibody does not contain a signal peptide but only the mature portion of the light chain polypeptide.

5.2.5 The skilled person would also know with certainty which part of the amino acid sequences in SEQ ID NO: 3 and SEQ ID NO: 4 is the signal peptide, and which part of SEQ ID NO: 3 and which part of SEQ ID NO: 4 is present in the mature antibody for the reasons set out in paragraphs 5.1.8 to 5.1.11 above.

5.3 *The cleavage site in SEQ ID NOs: 3 and 4 is certain*

5.3.1 In point (b) of paragraph 1.1.2 of the decision, the examining division does not contest that there may be usual starting and/or ending amino acids for a leader (signal) peptide and for the mature form of the light chain of an antibody. However, the examining division notes that:

> "*These usual amino acids or sequences amino acids are not present in totality of the light chains of antibodies.*" [emphasis added]

5.3.2 We are not, though, concerned with the totality of light chains of antibodies, but only with one specific antibody light chain. That light chain sequence is shown in SEQ ID NO: 4 of the application, and the variable portion of that light chain sequence is shown in SEQ ID NO: 3 of the application.

5.3.3 It is without doubt that SEQ ID NO: 3 and SEQ ID NO: 4 contain the ending amino acids for a light chain leader peptide (GARC) at positions 19-22, adjacent to the starting amino acids for the mature form of the light chain (DIQM) at positions 23-26. As explained in paragraphs 5.1.9 and 5.1.10 above with reference to the declarations, D14 and D32, in *all* cases where these sequences occur, they are at the boundary of the signal peptide and the mature sequence.

5.3.4 Given the presence in SEQ ID NO: 3 and SEQ ID NO: 4 of an N-terminal methionine followed by a stretch of mainly hydrophobic amino acids, and the usual ending amino acids of the signal peptide immediately adjacent

to the usual starting amino acids of the mature light chain, the skilled person would know with 100% certainty that a signal peptide is present in SEQ ID NO: 3 and SEQ ID NO: 4 and where the cleavage site between the signal peptide and the mature polypeptide lies.

5.3.5 It is irrelevant whether other light chains of other antibodies contain different signal peptide ending amino acids and different mature polypeptide starting amino acids.

5.3.6 The examining division also points out in point (b) of paragraph 1.1.2 of the decision that the evidence of common general knowledge we have filed refers to usual or common features of a leading peptide or a mature antibody sequence, but do not exclude completely the possibility of deviations from these features.

5.3.7 However, there is no deviation in so far as the particular sequences shown in SEQ ID NO: 3 and SEQ ID NO: 4 are concerned. SEQ ID NO: 3 and SEQ ID NO: 4 contain the ending amino acids for a light chain leader peptide (GARC) at positions 19-22, adjacent to the starting amino acids for the mature form of the light chain (DIQM) at positions 23-26 which, in *all* antibody light chain polypeptides in which they occur, are at the boundary of the signal peptide and the mature sequence (see D14 and paragraphs 5.1.9 and 5.1.10 above). Therefore, the exact length of the leader peptide in SEQ ID NO: 3 and SEQ ID NO: 4 can be determined with absolute certainty.

5.4 *An antibody would never comprise all of SEQ ID NO: 3 or 4*

5.4.1 In point (c) of paragraph 1.1.2 of the decision, the examining division considers that:

> *"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. This is particularly true in the present case since the application relates to the field of engineered antibodies and not*

of naturally occurring human antibodies (such as those mentioned in the Kabat document mentioned in D14)."

5.4.2 However, the skilled person would have no reason to think that the applicant intended to protect an antibody comprising a light chain signal peptide, and the skilled person would exclude this possibility with certainty.

5.4.3 The skilled person would know from his common general knowledge that engineered monoclonal antibodies comprising a full-length heavy chain and a full-length light chain having predetermined amino acid sequences are produced by recombinant expression in transfected cells (see the third Andrien declaration (D32), paragraphs 15 to 17, and D33, page 45, right column, page 46, second paragraph of section 5.1.2 and section 5.3). The application also explicitly discloses that monoclonal antibodies may be produced in transfected cells, such as CHO cells and NS0 cells (page 19, lines 6 to 7 of the application).

5.4.4 As clearly depicted in the prior art textbook Alberts (D20), hydrophobic signal peptides remain in the ER membrane and are cleaved off from the portions of polypeptides that form mature proteins by a peptidase. Thus, the skilled person would know, from his common general knowledge, that during antibody production in a cell the hydrophobic signal peptide of an antibody light chain remains in the ER membrane and is cleaved from the portion of the light chain polypeptide that forms part of the mature antibody by a peptidase. This is the same regardless of whether the antibody is naturally occurring or is an engineered antibody. The peptidase that cleaves the antibody polypeptide recognises the amino acid sequence at its cleavage site and does not distinguish between a naturally occurring polypeptide and an engineered polypeptide (see also D19).

5.4.4.1 Thus, contrary to the conclusion of the examining division, the skilled reader would have immediately excluded the possibility that the applicant sought to protect an antibody having a light chain consisting of the whole of SEQ ID NO: 3 or 4.

5.5 *The cleaved signal peptide would not be reattached*

5.5.1 In point (d) of paragraph 1.1.2 of the decision, the examining division considers that:

> *"It cannot be excluded with certainty that after production of the mature antibody in the transfected cells (which is only one possible way of producing the claimed antibody – see page 19 of the application), a sequence of amino acids is attached to the N-terminal amino acid."*

5.5.2 Here, the examining division appears to accept that a mature antibody produced in transfected cells will not contain a signal peptide, and to suggest that the skilled person might consider that after the mature antibody has been produced in transfected cells, the signal peptide is re-attached to the N-terminus of the light chains in the mature antibody.

5.5.3 There is no disclosure in the application to suggest that this is desirable or even possible. There is no example of this having been done in the art, and a skilled person reading the application in light of his common general knowledge would not contemplate this possibility (see third declaration of Dr Andrien (D32), paragraph 19).

5.5.4 Indeed, the application is concerned with the treatment of PNH using a therapeutic antibody that binds to C5. It would be common general knowledge to the skilled person that the immunogenicity of therapeutic antibodies should be minimised, and that to achieve this the antibodies need to mimic naturally occurring human antibodies (see D30, section headed "Alternative Forms of Antibodies" spanning pages 7 and 8, and the first paragraph of page 8 in particular; and D33, page 34, left column, particularly, first paragraph lines 9-21, second paragraph, and third paragraph, lines 1-2).

5.5.5 Naturally occurring human antibodies do not contain a signal peptide. Therefore, the skilled person would not for a moment consider that the applicant intended that a signal peptide that is naturally cleaved from the precursor light chain polypeptide during antibody production might

later be re-attached to the mature antibody (see third Andrien declaration (D32), paragraph 19).

5.5.6 The examining division is also incorrect that the application discloses that the claimed antibody could be produced other than in transfected cells. Page 19 of the application does not disclose other methods of producing therapeutic antibodies such as the claimed antibody. At page 19, lines 6 to 13 of the application, it is stated that:

> *"In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier 'monoclonal' indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., Nature 256:495-497 (1975), or may be made by recombinant methods (see e.g., U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al. Nature 352:624-628 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example."*

5.5.7 The skilled person would know that the hybridoma method is not suitable for producing an engineered full-length monoclonal antibody comprising a full-length heavy chain and a full-length light chain having predetermined amino acid sequences (see the review article D33, page 34, left column, first paragraph). However, the hybridoma method is at any rate a cell-based method that would inevitably result in cleavage of the signal peptide during antibody production (see D32, paragraph 16).

5.5.8 Further, recombinant methods involve expression of the antibody in transfected cells (see D33, page 46, section 5.3).

5.5.9 Also, phage antibody libraries contain only antibody fragments and are used for screening those antibody fragments. Phage antibody libraries are not used for the production of full-length antibodies (see D33, sections 3.2.3 and 3.2.3.1 at pages 37-39; and D32, paragraph 16).

5.5.10 There is, therefore, no disclosure in the application to suggest that the claimed antibody can be produced other than in cells, which, as discussed above, would always result in cleavage of the signal peptide at the known peptidase cleavage site present in SEQ ID NO:3 and SEQ ID NO:4 (see D32, paragraph 17).

5.6 *Summary: No added matter*

5.6.1 In conclusion, the skilled person would know from his common general knowledge that the claimed antibody must be produced in cells, and that the signal peptide in the antibody light chain polypeptide is always cleaved during antibody production in a cell. He would also immediately recognise exactly where in SEQ ID NO:3 and SEQ ID NO: 4 the cleavage site between the signal peptide and mature polypeptide lies.

5.6.2 Accordingly, the skilled person would know with certainty that the definition in the application of an antibody as comprising a light chain that consists of SEQ ID NO: 4 or the light chain variable region that consists of SEQ ID NO: 3 is an error, and that the antibody would comprise only the part of SEQ ID NO: 3 or SEQ ID NO: 4 beginning at amino acid 23.

5.6.3 Thus, when the present application is considered on the basis of its own merits in the light of the common general knowledge of the skilled person, it is immediately evident that nothing else was intended by the applicant other than what is offered as the correction to the definition of the light chain amino acid sequence of the antibody. The correction is therefore allowable under Rule 139 EPC.

5.6.4 Given that the correction is allowable under Rule 139 EPC, it follows that the Main Request meets with the requirements of Articles 76(1) and 123(2) EPC.

5.7 *Basis for Auxiliary Requests*

5.7.1 The comments above in relation to added matter apply to Auxiliary Request 6 (Auxiliary Request 2 considered by the examining division).

5.7.2 The examining division set out in paragraph 2.1 of the decision its view that the claims of Auxiliary Request 1 considered by the examining division (now presented as Auxiliary Request 4), which also apply to Auxiliary Request 3 considered by the examining division (now presented as Auxiliary Request 10), add subject matter. In claim 1 of these auxiliary requests, the antibody is defined as comprising

> *"the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4".*

5.7.3 At page 4, line 32 of the application as filed (and the parent application), it is stated that:

> "*In certain embodiments, said antibody is eculizumab.*"

5.7.4 The heavy and light chain amino acid sequences of eculizumab are shown in SEQ ID NO: 2 and SEQ ID NO: 4, respectively, at page 44 of the application as filed (and the parent application).

5.7.5 Hence, there is direct and unambiguous basis for the claimed antibody that comprises the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the amino acid sequence of the light chain shown in SEQ ID NO: 4.

5.7.6 The skilled person reading the application would immediately recognise that the amino acid sequence shown in SEQ ID NO: 4 shows both the signal peptide sequence and the sequence of the light chain that is present in eculizumab. Accordingly, he would understand that eculizumab comprises the light chain sequence shown in SEQ ID NO: 4.

5.7.7 The language used in claim 1 of Auxiliary Requests 1 and 3 considered by the examining division (now presented as Auxiliary Requests 4 and 10) therefore describes what is disclosed in the application and, although the words "shown in" are not used in the originally filed application or parent

application, the inclusion of these words in the claim does not result in the claim describing subject matter that extends beyond the content of the original application and parent application.

6 **Novelty**

6.1 In section 5 of the decision, the examining division states that the present application does not meet the requirements of Article 54 EPC because eculizumab is considered to have been available to the public before the filing date of the present application. Contrary to the examining division's conclusion, however, the claimed subject matter was not available to the public before the filing date and the claimed subject matter is therefore new.

6.2 The examining division's decision was based on a finding of lack of novelty over the prior use in clinical trials, D1 to D5, D10 and D11. Each of these matters is discussed below. The applicant also refers to the earlier submission of 5 July 2019 which provided a detailed discussion in relation to novelty. These arguments form a part of the applicant's Grounds of Appeal and are provided herewith as Annex 1.

6.3 In addition to the specific objections mentioned above, various other questions in relation to the differences between the claimed invention and the art were raised by the examining division earlier in the present proceedings. These matters were not discussed during the oral proceedings and do not form a part of the examining division's decision. They are therefore not addressed in detail here. However, for completeness, in this regard the applicant refers to the 5 July 2019 submission provided herewith.

6.4 *Prior use of eculizumab in clinical trials*

6.4.1 In section 5.1.1 of the decision, the examination division set out its opinion that scientists working with eculizumab prior to the filing date of the present application were not bound to confidentiality with regard to

the structure of eculizumab. For this reason, the examining division considered that the prior use of eculizumab in clinical trials before the filing date of the present application was prejudicial to novelty.

6.5 *Summary of Evidence relating to Clinical Trials*

6.5.1 Before considering the examining division's position in further detail, it is useful to set out a summary of the evidence relevant to the prior use of eculizumab during clinical trials which is on file in these proceedings.

6.5.2 The principle evidence is D31, the declaration by Dr Michael Bombara. Dr Bombara is executive director of clinical business operations - R&D of Alexion Pharmaceuticals, and acted as project lead for a number of eculizumab clinical trial studies. Dr Bombara provides a detailed explanation of the various contracts and agreements associated with each eculizumab clinical trial and the confidentiality provisions which were in place. By way of example, Dr Bombara focusses particularly on trial C02-001 carried out at Leeds Teaching Hospital.

6.5.3 Exhibits to Dr Bombara's declaration include, in particular:

Exhibit C: master clinical study agreement (MCSA), Leeds Teaching Hospitals NHS Trust;

Exhibit D: Exhibit II to the MCSA for trial C02-001 carried out at Leeds Teaching Hospital;

Exhibit E: study protocol for trial C02-001; and

Exhibit H: example of drug logging form.

6.5.4 Supporting evidence is also provided by:

D12: declaration of Leonard Bell, a co-inventor of the invention, in which Dr Bell confirms that clinical trials in relation to eculizumab were carried out prior to 15 March 2007, but only under strict control and conditions of confidentiality.

D13: declaration of Laural Boone, patent counsel at Alexion Pharmaceuticals, providing a summary of the clinical trial studies carried out in relation to eculizumab, as well as evidence of the confidentiality requirements set forth in the clinical study protocols.

D22: a redacted study protocol for trial C04-001

D23: a redacted protocol for trial C02-002

6.5.5 We also provide with our Grounds of Appeal the following new documents:

D34: declaration of Mr Andrew Webb providing legal interpretation of the clinical trial study agreements; and

D35: declaration of Dr Anita Hill, who was involved in some of the clinical trials carried out at Leeds Teaching Hospitals in relation to eculizumab.

6.6 *Eculizumab Not Made Available to Public by Prior Use at Clinical Trials*

6.6.1 It is established jurisprudence before the Boards of Appeal of the EPO that the following need to be clarified to determine whether an invention has been made available to the public by prior use: (1) when the prior use occurred; (2) what was made available to the public through that use; and (3) the circumstances of the use, i.e. where, how and by whom the subject matter was made public through that use (see Case Law of the Boards of Appeal Chapter I.C, 3.2.4). In the present case these questions can be answered as follows:

When: eculizumab was provided to a number of institutions for the purpose of conducting specific clinical trials before the filing date of the application (D12 declaration of Leonard Bell, paragraph 2; D13 declaration of Laural Boone, paragraphs 3 to 6).

What: the research institutes were provided with eculizumab having the relevant light and heavy chain sequences (D13 declaration of Laural Boone, paragraph 6).

Circumstances: the supply of the drug to scientists was not a commercial supply but rather a supply specifically for use in the context of the proposed clinical trials, and the supply was governed by agreements imposing conditions of confidentiality on the scientists. The supply did not lead to eculizumab being made available to the public.

6.6.2 The Boards of Appeal have previously decided that medical professionals and hospital staff involved in the operation of clinical trials are, in general, considered to be under an obligation of confidentiality. Decision T152/03 for example, concerned a potential prior use of a device referred to as a Guglielmi Detachable Coil (GDC). In that decision, the Board addressed the question of confidentiality in section 3.4, stating that:

> "*In this field there is a prima facie assumption that any person involved in a medical process is obliged to confidentiality, given the need for patient confidentiality and the need to protect the development and testing of prototype devices.*"

6.6.3 In a similar decision in T906/01, the Board held that clinical tests performed before marketing were implicitly confidential:

> "*Usually the development and testing phases of such products or devices are necessarily surrounded by secrecy as long as said products or devices have not been approved and commercialised (see a nearly similar situation in T818/93, point 4.1, unpublished). Therefore, even without the production of more specific evidence on behalf of the respondent, the Board is of the opinion that the clinical tests performed on the Isola spinal system under the conduct and responsibility of Dr Flatley conferred to the overall operation an implicit obligation of confidentiality which had to be extended to the whole team involved in said operation.*"

6.6.4 It is therefore established in the case law that clinical trials are considered to be confidential amongst the scientists carrying out the trial. This general principle would, of course, apply to the present situation where clinical trials were carried out in respect of eculizumab.

6.6.5 In the present case, moreover, a number of explicit agreements were in place in relation to each trial with eculizumab. These agreements limited exactly how the trial drug (eculizumab) should be used in respect of the trial, and confirm that the investigators conducting the trial were under an obligation of confidentiality with respect to information they were provided, and in respect of discoveries which were made, during the trial.

6.6.6 In support of this matter, we provide here D34, a declaration of Mr Andrew Webb, a UK Solicitor having expertise in advising on contracts relating to technical and commercial information. Mr Webb was asked to review the various agreements and evidence provided in relation to the eculizumab clinical trials in these proceedings and to provide his expert opinion on any obligations which these agreements imposed on the scientists carrying out the clinical trials, with regard to the public disclosure of the sequence of eculizumab.

6.6.7 It is noted that Mr Webb's declaration refers explicitly to the related proceedings on the sister divisional application no. 16172335.8 (J A Kemp ref: N411599EP-A). However, the issues raised by the examining division, and the documents cited in proceedings, are identical in both cases. For completeness the document references in both cases are as follows:

Cited document in present proceedings	Reference in Webb declaration
D31: Bombara declaration	D36
D12: Bell declaration	D31
D13: Boone declaration	D13
D22: redacted protocol for trial C04-001	D22
D23: redacted protocol for trial C02-002	D23

6.6.8 Mr Webb summarises at paragraph 2.2 of his declaration (i) the obligation of the investigators to use the study drug only for the purposes provided and (ii) the restrictions placed on investigators with regard to information discovered during the trial:

> *"The supply of eculizumab for clinical trials was for use in those trials in accordance with the trial protocol and the recipients were not free to use the product provided for any other purpose. The recipients were not free in equity and law to use the product for conducting sequence analysis and there is no evidence that they did so in breach of their obligations. That is sufficient to overcome the Examining Division's objections;*
>
> *Even if the recipients had analysed the product, any information the recipients discovered concerning the product accrued to Alexion and was subject to restrictions on use and disclosure."*

6.6.9 Evidence to support these conclusions is provided in section 5 of the declaration. In relation to point (i) above, Mr Webb refers to the study protocol provided as Annex E to Bombara declaration D31. The study protocol sets out how the scientists are permitted to use the drug which is supplied for the trial. For instance, section 6.2 of the protocol sets out how the scientists should store the study drug, noting that *"eculizumab must be stored in a secure, limited access storage area."* Further, section 6.6 of the protocol relates to drug accountability and places particular requirements on the study investigators to ensure that all drug is properly logged and accounted for. An example of a drug logging form used in conjunction with an eculizumab clinical trial is provided as D31 Exhibit H (see also paragraphs 12 and 13 of D31).

6.6.10 This evidence, together with the nature of the agreements as a whole, leads to the conclusion set out at paragraph 5.9 of Mr Webb's declaration:

> *"eculizumab is provided for a specific limited purpose, to carry out the tests in accordance with the test protocol. This does not permit the recipient to use the supplied product for any other purpose. The*

recipient is not free to use the product for any other purpose and must account for all product provided."

6.6.11 It is therefore clear that the scientists involved in the clinical trials in relation to eculizumab were not free to experiment with the study drug, or to analyse its sequence.

6.6.12 Moreover, even if the scientists had, contrary to the agreements relating to the trial, conducted any analysis of the sequence of eculizumab, that newly discovered information would belong to Alexion and the scientists themselves would not be free to disclose it to the public (see paragraph 2.2, second point of D34).

6.6.13 The evidence which gives rise to this conclusion is discussed in section 5 of D34. In particular, clauses 2 and 7 of the Master Clinical Study Agreement (D31, Exhibit C) specify that:

"*Clause 2 Data and Inventions - …. All data, inventions, and discoveries …which… relates to a Study Drug shall belong solely and exclusively to Alexion.*

Clause 7 Confidentiality and Publication - Institution agrees and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study…..data, inventions and discoveries which …..relate to a Study Drug. The preceding obligations shall not apply to information, data, inventions, or discoveries which have entered the public domain…"

6.6.14 Therefore, even if new information such as the sequence of eculizumab had been discovered during the clinical trials, the scientists were bound by contract to keep such information confidential. The circumstances surrounding the prior use therefore clearly establish that the sequence of eculizumab was not made available to the public through its use by scientists in carrying out clinical trials.

6.7 *Errors in examination division decision*

6.7.1 The examining division erred in a number of respects in reaching its decision. Firstly, the examining division indicates at paragraph 5.1.1 of the decision:

> "*However, the examining division is of the opinion that confidentiality agreements cannot relate to information that is (believed to be) in the public domain. The scientists working with eculizumab were under the impression that the structure of eculizumab had been submitted to CAS. Hence, they were not bound confidentiality with regard to the structure of eculizumab*".

6.7.2 As the examining division explains, the structure of eculizumab which was submitted to CAS in February 1999 contained a number of errors and the correction of these errors was not obvious. Accordingly, the correct sequence of eculizumab, as presently claimed, was not made available via CAS prior to the filing date of the present application. The examining division asserts, however, that because the scientists may have *thought* the sequence was in the public domain, they would no longer be bound to confidentiality with regard to the sequence. However, the examining division does not provide any legal support for reaching this conclusion and their conclusion is not correct.

6.7.3 The law surrounding the confidentiality provisions to which the examining division refers is discussed in Section 8 of Mr Webb's declaration. Mr Webb explains that there are a number of ways in law in which an obligation of confidentiality may be imposed. In the present case, the obligation is imposed by the contracts entered into between Alexion and the institutions carrying out the clinical trials. The information which is confidential is therefore that set out in the contracts themselves, and includes "*data, information and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug*".

6.7.4 Mr Webb acknowledges that the law recognises that public policy considerations can make overbroad contractual confidentiality agreements unenforceable (para 8.3, D34). However, Mr Webb considers that the contract in the present case recognises this and limits the scope

of the confidentiality clause accordingly. It does not, however, exclude from the confidentiality clause information which the scientists would have *thought* was in the public domain. Rather, it excludes (as is normal in such contracts) information, data, inventions or discoveries *which have* entered the public domain. The sequence of eculizumab *had not* entered the public domain and therefore did not fall within this exclusion.

6.7.5 Contrary to the examining division's position, Mr Webb explains that

> "*the question of whether information is in the public domain is a question of fact, to be determined by evidence*" (para 8.6).

6.7.6 The examining division does not contest that the structure of eculizumab which was submitted to CAS contained errors, and that the correction of these errors was not obvious (5.1.1 of examining division decision). However, the examining division provides no evidence as to when or how the correct sequence of eculizumab was put into the public domain, such that it would be excluded from the confidentiality obligations set out in the exemplary MCSA under clause 7.

6.7.7 Despite this lack of evidence, the examining division considers the sequence of eculizumab to be excluded from the confidentiality provision because, as a matter of law, something which one party *believed to be* in the public domain should be excluded. No support is provided for this legal position. It is not one recognised by Mr Webb, an expert in this area (para 8.7, D34).

6.7.8 The examining division was *not correct* to conclude that the erroneous disclosure of the sequence to CAS rendered the confidentiality provisions null and void with regard to the sequence. Thus, even if the scientists carrying out the clinical trials with eculizumab had been (or came to be) in possession of the correct eculizumab sequence during the course of the trial, per the terms of the agreements, that information would have to remain confidential, belonging to Alexion.

6.7.9 A second error in the examining division decision appears in their statement that:

"the scientists working with eculizumab… could have performed a sequencing of the antibody with the general knowledge available in the art without technical or legal constraints"

6.7.10 Regarding the technical constraints which prevented the scientists from sequencing the antibody, the applicant provided comments on this matter at pages 10 to 11 of the submission of 15 August 2018 (Annex 2) which are further relied on here. In brief, the applicant considers that it would not have been routine in the art to obtain the full sequence of eculizumab at the filing date of the present application. The examining division did not comment on this argument at all in their decision, despite the matter having been raised in oral argument at the oral proceedings. No reasoning is given as to why the examining division considers that the structure of eculizumab could have been reproduced as required by G1/92.

6.7.11 Regarding the legal constraints which prevented the scientists from sequencing the antibody, as explained in detail above, the scientists involved in the trials were operating in accordance with agreements which did not allow the sequencing of eculizumab.

6.8 *Summary: Prior Use*

6.8.1 In summary, the evidence on file in the present proceedings supports the fact that the scientists who carried out clinical trials before the filing date of the present application were, according to the contracts entered into, required to use eculizumab only for the specified purpose of carrying out the clinical trial, and were not free to sequence eculizumab.

6.8.2 Further, even if they had obtained the correct sequence, any such new information obtained during the trial would have belonged to Alexion and the scientists would not have been free to disclose that information publically.

6.8.3 Finally, the examining division was incorrect as a matter of law to suggest that the presence of the incorrect eculizumab sequence on the CAS register has any impact on the restrictions on use and disclosure imposed

by the clinical trial contracts. Eculizumab was not therefore made available to the public through carrying out clinical trials before the filing date of the present application.

6.9 *Differences between claim and D1 to D5*

6.9.1 At 5.1.2 of the decision, the examining division also states that the application lacks novelty over D1 to D5. However, the examining division fails to provide any indication as to where in D1 to D5 a disclosure of the sequence of eculizumab can be found.

6.9.2 D1 to D5 refer to eculizumab by name. However, none of these documents includes any structural information for eculizumab. It is understood that the examining division accepted that none of D1 to D5 themselves included structural information for eculizumab. Nevertheless, in case there is any doubt in this regard, we refer to the applicant's submission of 5 July 2019 (Annex 1) which discusses in full the disclosure of D1 to D5 at pages 2 to 3.

6.9.3 The applicant understands that the examining division took the view that because the structure of eculizumab was known via the prior use in clinical trials (a matter which is disputed by the applicant), this structure could be "read in" to the disclosures of D1 to D5 such that the structural information lacking in those documents is rectified.

6.9.4 This is an approach which is not in conformity with the EPC. A finding of lack of novelty must be based on a ***single*** disclosure. As set out in the Guidelines for Examination at G-VI, 1, it is not permissible to combine separate prior art documents in forming an objection of lack of novelty. Thus, the examining division's combination of the information from the alleged prior use, with that of D1 to D5, was not permissible in a lack of novelty objection.

6.9.5 It is possible (though not stated) that the examining division considered that the structure of eculizumab was implicit in D1 to D5. However, given that the structure of eculizumab was not known in the art prior to

the filing date, this cannot be the case. Moreover, even if the structure *had* been made available by prior use, this would not make the structure of eculizumab implicit in the sense that a skilled reader could infer it directly and unambiguously from any of D1 to D5 (the standard referred to in the Case Law of the Boards of Appeal, I-C, 4.3). Rather, a combination of one of D1 to D5 with the prior use would have been needed.

6.9.6 Therefore, none of D1 to D5 provides an enabling disclosure of eculizumab since there is no information on the sequence of the antibody in any of these documents. The claims are therefore novel over D1 to D5.

6.10 *Novelty over D10*

6.10.1 The examining division states at 5.1.3 of the decision that the application lacks novelty over D10 because in earlier proceedings Alexion's representative had stated that the structure of the antibody had been made available to the public on 14 February 1999 (see D9 in the present proceedings). The 14 February 1999 disclosure is the CAS database entry which is now known to have included a *different* sequence from that of eculizumab itself.

6.10.2 The examining division were incorrect to reach this conclusion and does not provide proper basis for a finding of lack of novelty.

6.10.3 A finding of lack of novelty over a prior art document must be based on a consideration of the disclosure of that document. In the present case, instead of pointing to the passages in D10 which describe the sequence of eculizumab, the examining division rely on a statement made about the disclosure of D10 in separate proceedings. Such a statement is not relevant to the question of novelty, which is matter of fact, and turns *only* on the disclosure of the document in question.

6.10.4 Contrary to the examining division's comments, D10 does not provide an enabling disclosure of eculizumab. Detailed reasoning for this conclusion is found in the applicant's 5 July 2019 submission (Annex 1, pages 5 to 7).

6.11 *Novelty over D11*

6.11.1 The examining division makes a similar error in relation to the argument of lack of novelty over D11. At 5.1.4 of the decision, the examining division does not point to the passages in D11 which disclose the sequence of eculizumab. Rather, it relies on a statement in the description of the present application found at page 16, lines 24-25: "*These antibodies disclosed in U.S. Patent 6,355,245 [D11] include a preferred whole antibody (now named eculizumab).*"

6.11.2 However, it is necessary to look at what D11 actually discloses to determine whether or not there is a lack of novelty. As discussed in detail in the submission of 5 July 2019 (Annex 1, pages 3 to 4), the only disclosure relating to full length antibodies in D11 is a paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g. a VL and a VH region), Fds and light chains disclosed therein may be combined with constant region domains to form full length antibodies. This passage does not provide a disclosure of the subject matter of present claim 1.

6.11.3 The statement in the present application at page 16, lines 24 to 25 should therefore correctly be interpreted as meaning that D11 discloses a genus of antibodies, and not that the sequence of this specific antibody is specifically disclosed in D11. It is a matter of fact that the sequence of eculizumab *is not* disclosed in D11. The claimed subject matter is therefore novel over D11.

6.12 *Novelty of Auxiliary Requests*

6.12.1 The comments above in relation to novelty apply to all of the Auxiliary Requests.

7 Inventive Step

7.1.1 The present application is concerned with providing treatments for paroxysmal nocturnal hemoglobinuria (PNH), and claim 1 is directed to an antibody that binds C5. D1 can be considered to be the closest prior art document because it is concerned with treating PNH and describes a clinical trial using an anti-C5 antibody.

7.1.2 The antibody used in D1 is referred to as "eculizumab". D1 does not disclose the sequence of "eculizumab", but cites D7 (Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996) and D11 (U.S. Pat. No. 6,355,245) as describing suitable anti-C5 antibodies including "eculizumab":

> *"Methods for the preparation of h5G1.1-mAb, h5Gl.l-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab."* (D1, page 5, left column last two lines to right column line 8).

7.1.3 D11 does not disclose the full sequence of any humanised antibody. The only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how:

> *"matched pairs of the variable regions (e.g., a VL and a VH region) of the various antibody molecules, Fds, and light chains described above may be combined with constant region domains to form full length antibodies of the invention. Particularly preferred constant regions for this purpose are IgG constant regions, which may be unaltered, or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG 4."*

7.1.4 D7 provides VL and VH variable regions for the h5G1.1-mAb antibody (Figure 1 of D7)[1] and describes an IgG4 isotype antibody:

> "*Having demonstrated the effective humanization of the 5G1.1 variable regions, an intact humanized antibody (IgG4 isotype) was constructed and produced in 293-EBNA cells.*" (D7, page 1396, sentence spanning columns).

7.1.5 No other full length antibodies are described in D7. Therefore, the teaching in D1 would have led the skilled person to treat PNH using an antibody having the VL and VH sequences disclosed in D7 which has an IgG4 heavy chain constant region.

7.1.6 In contrast, the antibody of claim 1 does not have an IgG4 constant region, but has an engineered heavy chain constant region. The amino acid differences between the IgG4 heavy chain constant region and the engineered heavy chain constant region of the antibody of claim 1 are shown in the Bell declaration (D12) in paragraph 5. There are four amino acid differences in the CH1 domain, six amino acid differences in the hinge domain and four amino acid difference in the CH2 domain.

7.1.7 The technical effects of these differences are reduced immunogenicity and increased plasma half-life. As explained in paragraphs 6 and 7 of D12, the differences in the heavy chain constant region between an IgG4 antibody of D7 and the claimed antibody result in the antibody having (i) reduced binding to Fcγ receptors (shown experimentally in the report filed as D18) such that immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway are minimised; and an increased plasma half-life (shown experimentally in the report filed as D29) so that it does not need to be administered to patients as frequently.

7.1.8 The objective technical problem in view of D1 is the provision of an antibody for therapeutic use in treating PNH having a reduced ability to

[1] The VL sequence in D7 contains is different from the VL sequence of the claimed antibody (eculizumab) at position 38 - eculizumab contains Gln(Q) at this position and not Arg(R). This is explained in our letter of 16 January 2018 (Annex 3).

stimulate undesirable immune responses and an increased plasma half-life compared to the IgG4 antibody of D7.

7.2 *Provision of the Claimed Antibody is not Obvious*

7.2.1 The solution is solved by the presently claimed antibody which has an engineered heavy chain constant region that differs from the IgG4 heavy chain by four amino acids in the CH1 domain, six amino acids in the hinge domain and four amino acids in the CH2 domain compared to the IgG4 antibody of D7. This solution would not have been obvious to the skilled person at the filing date.

7.2.2 D1 is silent as to the effects of the heavy chain constant region on the properties of the antibody.

7.2.3 D11 discloses that constant regions may be constructed of a mixture of constant domains, but does not teach or suggest the specific differences between the IgG4 constant region amino acid sequence and the constant region sequence of the claimed antibody. Also, D11 does not mention any reasons for deviating from the unaltered, naturally-occurring IgG constant regions.

7.2.4 D7 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> *"The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)." (D7, page 1399, right column, second paragraph, lines 15-25)."*

7.2.5 The skilled person would have had no motivation to use an antibody other than the IgG4 antibody described in D7 in view of the report in D1 of the successful treatment of PNH, and the teaching in D7 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only one known allotype, precluding an immune response against the constant region of the antibody.

7.2.6 In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D7 because he would be concerned about the immunogenicity of the therapeutic antibody, and D7 teaches that the IgG4 isotype is ideal in this regard. The skilled person would not have tried to reduce unwanted immune responses by engineering the heavy chain constant region to have a non-natural sequence.

7.2.7 Also, at the filing date of the application, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D30, table 1). Significantly, at the filing date of the application, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market.

7.2.8 The skilled person would therefore have had no motivation to alter the constant region of the antibody disclosed in D7 (which is the antibody to which D1 refers).

7.2.9 Furthermore, the advantageous increased half-life exhibited by the claimed antibody would not have been expected based on the teaching of any of the cited documents. Indeed, in paragraph 8 of D31, it is explained that the advantageous properties of the claimed antibody over the antibody of D7 (which is the antibody to which D1 refers) were surprising and unpredictable.

7.2.10 Accordingly, the solution to the problem in view of D1 of providing an antibody having improved properties (a reduced ability to stimulate undesirable immune responses and an increased plasma half-life) compared to the IgG4 antibody of D7 (which is the antibody to which D1

refers) for therapeutic use in treating PNH provided by the present claims would not have been obvious to the skilled person at the filing date. Accordingly, the claims involve an inventive step.

7.3 *Inventive Step of Auxiliary Requests*

7.3.1 The comments above in relation to inventive step apply to all of the Auxiliary Requests.

J A • K E M P

BY ONLINE FILING

The European Patent Office
80298 Munich
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19 June 2020

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

Further to our notice of appeal dated 24 March 2020, we are now filing our grounds of appeal. We attach the following documents:

- Statement of Grounds of Appeal;
- Main Request and Auxiliary Requests 1-11;
- tracked changes versions of Auxiliary Requests 1-6, showing the changes relative to the Main Request;
- tracked changes versions of Auxiliary Requests 7-11, showing the changes relative to Auxiliary Requests 1-5, respectively;
- D32: Third Declaration of Dr Bruce Andrien;
- D33: Review article, Laffly & Sodoyer, "*Monoclonal and Recombinant Antibodies, 30 Years After…*", Human Antibodies (2005), 33-55;
- D34: Declaration of Mr Andrew Webb;
- D35: Declaration of Dr Anita Hill;
- Annex 1: Submission of 5 July 2019;
- Annex 2: Submission of 15 August 2018; and
- Annex 3: Submission of 16 January 2018.

We request that the Decision of the Opposition Division dated 11 February 2020 be set aside and the application be allowed on the basis of the Main Request or, failing that, on the basis of one of the Auxiliary Requests.

Should the Board of Appeal envisage any decision other than allowance of the application on the basis of the Main Request, oral proceedings are requested.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
FOR J A KEMP LLP
ASSOCIATION NO. 811

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Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Statement of grounds of appeal	Statement of Grounds of Appeal N411599EP-B.PDF	APPEAL-GRDS-1.PDF
2	Letter relating to the Appeal procedure	Auxiliary Request 1 (clean) N411599EP-B.PDF	APPEAL-LETT-6.PDF
3	Letter relating to the Appeal procedure	Auxiliary Request 1 (tracked) N411599EP-B.PDF	APPEAL-LETT-7.PDF
4	Letter relating to the Appeal procedure	Auxiliary Request 2 (clean) N411599EP-B.PDF	APPEAL-LETT-8.PDF
5	Letter relating to the Appeal procedure	Auxiliary Request 2 (tracked) N411599EP-B.PDF	APPEAL-LETT-9.PDF
6	Letter relating to the Appeal procedure	Auxiliary Request 3 (clean) N411599EP-B.PDF	APPEAL-LETT-10.PDF
7	Letter relating to the Appeal procedure	Auxiliary Request 3 (tracked) N411599EP-B.PDF	APPEAL-LETT-11.PDF
8	Letter relating to the Appeal procedure	Auxiliary Request 4 (clean) N411599EP-B.PDF	APPEAL-LETT-12.PDF
9	Letter relating to the Appeal procedure	Auxiliary Request 4 (tracked) N411599EP-B.PDF	APPEAL-LETT-13.PDF
10	Letter relating to the Appeal procedure	Auxiliary Request 5 (clean) N411599EP-B.PDF	APPEAL-LETT-14.PDF
11	Letter relating to the Appeal procedure	Auxiliary Request 5 (tracked) N411599EP-B.PDF	APPEAL-LETT-15.PDF
12	Letter relating to the Appeal procedure	Auxiliary Request 6 (clean) N411599EP-B.PDF	APPEAL-LETT-16.PDF
13	Letter relating to the Appeal procedure	Auxiliary Request 6 (tracked) N411599EP-B.PDF	APPEAL-LETT-17.PDF
14	Letter relating to the Appeal procedure	Auxiliary Request 7 (clean)	APPEAL-LETT-18.PDF

		N411599EP-B.PDF	
15	Letter relating to the Appeal procedure	Auxiliary Request 7 (tracked) N411599EP-B.PDF	APPEAL-LETT-19.PDF
16	Letter relating to the Appeal procedure	Auxiliary Request 8 (clean) N411599EP-B PXT.PDF	APPEAL-LETT-20.PDF
17	Letter relating to the Appeal procedure	Auxiliary Request 8 (tracked) N411599EP-B PXT.PDF	APPEAL-LETT-21.PDF
18	Letter relating to the Appeal procedure	Auxiliary Request 9 (clean) N411599EP-B.PDF	APPEAL-LETT-22.PDF
19	Letter relating to the Appeal procedure	Auxiliary Request 9 (tracked) N411599EP-B.PDF	APPEAL-LETT-23.PDF
20	Letter relating to the Appeal procedure	Auxiliary Request 10 (clean) N411599EP-B.PDF	APPEAL-LETT-24.PDF
21	Letter relating to the Appeal procedure	Auxiliary Request 10 (tracked) N411599EP-B.PDF	APPEAL-LETT-25.PDF
22	Letter relating to the Appeal procedure	Auxiliary Request 11 (clean) N411599EP-B.PDF	APPEAL-LETT-26.PDF
23	Letter relating to the Appeal procedure	Auxiliary Request 11 (tracked) N411599EP-B.PDF	APPEAL-LETT-27.PDF
24	Letter relating to the Appeal procedure	Main Request N411599EP-B.PDF	APPEAL-LETT-1.PDF
25	Annexes (other than cited documents) regarding appeal procedure	Annex 1 - Submissions of 5 July 2019 N411599EP-B.PDF	APPEAL-ANNEX-1.PDF
26	Annexes (other than cited documents) regarding appeal procedure	Annex 2 - Submissions of 15 Aug 2018 N411599EP-B.pdf	APPEAL-ANNEX-2.pdf
27	Annexes (other than cited documents) regarding appeal procedure	Annex 3 - Submissions of 16 January 2018 N411599EP-B.PDF	APPEAL-ANNEX-3.PDF
28	Letter relating to the Appeal procedure	D32 - Third Declaration of Andrien.pdf	APPEAL-LETT-5.pdf
29	Letter relating to the Appeal procedure	D33 Laffly and Sodoyer 2005.PDF	APPEAL-LETT-4.PDF
30	Letter relating to the Appeal procedure	D34 Declaration of Webb.PDF	APPEAL-LETT-3.PDF
31	Letter relating to the Appeal procedure	D35 - Declaration of Hill.pdf	APPEAL-LETT-2.pdf
32	Letter relating to the Appeal procedure	File Grounds of Appeal N411599EP-B PXT.PDF	APPEAL-LETT-28.PDF

	Payment	
1	Method of payment	Not specified

Signatures

Place:	**Oxford, GB**
Date:	**19 June 2020**
Signed by:	**Pamela Tuxworth 23470**
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Capacity:	**(Representative)**

MAIN REQUEST

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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Human Antibodies 14 (2005) 33–55
IOS Press

Monoclonal and recombinant antibodies, 30 years after...

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Abstract. In 1975, the hybridoma technology provided, for the first time, an access to murine monoclonal antibodies. During the two following decades, their high potential, as laboratory tools, was rapidly exploited, but *in vivo* applications were still very limited. Nowadays, antibodies, omnipresent in both diagnostic and research domains, are largely invading the domain of therapy. A wide array of novel technologies, including phage display and transgenic mice, to isolate fully human antibodies and engineer these molecules, has been implemented. The natural propensity, of the antibody molecules, to metamorphosis makes them an ideal response to new applications and therapeutic challenges. The present review is a tentative update of the different antibody "formats" and a walk through the techniques recently applied to antibody engineering. In addition it also addresses some specific issues such as the development of expression systems suitable for large-scale production of recombinant antibodies.

Keywords: Recombinant antibodies, engineering, immunotherapy, phage display, expression system

1. Introduction

As much as eighteen antibodies have been, to date, approved by the US Food and Drug Administration (FDA) for various therapeutic applications (Table 1): three murine, five chimeric, nine humanized and one human. Antibodies and their derivatives account for more than 30% of all biopharmaceuticals under current development. As a result of growth in existing markets for mAbs therapeutic and the opening of new ones, the global market is projected to increase to US$ 16,7 billions in 2008 [1,2]. Moreover, at least 130 mAbs are currently under clinical trials, including 55 humanized Abs and 37 fully human products. Additionally, new technologies including conjugated Abs and antibody fragments are expected to rise in importance (currently 19.7% of the antibody clinical pipeline) [2]. Monoclonal antibodies are set to play a significant role in the treatment of a wide number of indications in various therapeutic domains, such as oncology [3–5], asthma, transplant rejection, inflammatory, autoimmune, cardiovascular [6,7] or infectious diseases [8]. In cancer therapy, the goal is to induce, upon antibody administration, the direct or indirect destruction of cancer cells, by specific targeting the tumour (Rituximab®) or the vasculature that nourishes the tumour (Avastatin®). Numerous anti-inflammatory antibodies already on the market (Remicade®, Humira®) or under clinical trials (IDEC C9.1) have also shown the great potential of mAbs. Although the recombinant antibody technology allow a large diversity of potential applications such as sensitive detection (piezoimmunosensors) [9], prevention of dandruff [10], or removal of environmental contaminants [11]), this review will focus on the therapeutic potency of mAbs and their derivatives, illustrated by innovations progressively and continuously introduced during the past 30 years.

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2. Antibody engineering: A quest for humanity (chimerization, humanization, de-immunization)

In 1975, Georges J.F. Köhler and César Milstein developed and described the hybridoma technique for the generation of monoclonal antibodies [12] (Fig. 1), consisting in immortalizing mouse antibody-producing cells through fusion with tumour cells. Mouse hybridomas were the first reliable source of monoclonal antibodies (mAb), facilitating the large-scale production of murine mAb (Mumab) generated against any antigen. These first generations of murine mAb, as therapeutic agents, suffered a number of drawbacks: short serum half-life, insufficient activation of human effector functions and development of human anti-mouse-antibody (HAMA) responses in patients, especially when repeated administrations were necessary [13]. These problems have recently been overcome, to a large extent, using genetic engineering techniques. Different strategies have been developed to avoid, mask, or redirect this human immune response: 'chimerization' by fusion of mouse variable regions to human constant regions [14], 'humanization' [15] and 'de-immunization' by removal of T-cell epitopes [16,17] (Table 2).

2.1. Chimerization

In chimeric antibodies, the murine constant regions are replaced with human equivalent regions, on the basis that the constant region contributes a significant component to the immunogenicity. In addition, this allows for a better interaction with human effector cells and the complement system. This strategy led to therapeutic successes such as basiliximab (Simulect®: IgG1 anti-CD25, Novartis) or cetuximab (Erbitux®: IgG1 anti-EGFR, ImClone). Chimeric antibodies were perceived as less foreign, and therefore less immunogenic than mouse monoclonal antibodies, nevertheless human anti-chimeric antibody responses (HACA) have been observed (for example with infliximab/Remicade® [18]).

2.2. Humanization

In such a context, antibody humanization technology was developed, and made possible by the transfer of all xenogeneic CDRs (complementary determining regions) onto the framework of a human Ab. Different approaches of CDR-grafting have been tested according to the human template used as scaffold: sequences with known crystal structure [19–21], rearranged somatic sequences, unmodified germline sequences [22] or consensus sequence [23].

The first humanized Abs were constructed based on human sequences with known crystal structure, which permits the identification of residues contributing to the antigen binding. In the "best fit" strategy, the closest human sequence, usually rearranged, is used as a framework to receive the murine CDRs. Another approach for humanizing an Ab is to choose the closest human germline sequence [22]. Indeed, human Ab genes are formed *in vivo* by rearrangements of germline gene segments. Only later in B cell ontogeny, occurs a hypermutation process, tailoring the initial sequences to improve recognition of specific Ag. The body thus may be more tolerant to germline-encoded Abs. Also, clinical problems would be reduced if germline sequences were used for constructing humanized Abs. The consensus method utilizes variable light (V_L) and variable heavy (V_H) domain frameworks derived from the most common amino acid found at each position within a given human subgroup. Whatever the method, CDR-grafting might not result in the complete retention of antigen-binding properties because some framework residues can interact directly with antigen [24], or affect the conformation of CDRs loops [21]. In this case, the antibody must be engineered to fine tune the structure of the antigen-binding loops to restore high affinity. In the other hand, humanization of a xenogeneic Ab does not necessarily abolish the immunogenicity of the molecule all together, since the humanized Ab can still induce response against its xenogeneic CDRs. Not all residues within the CDRs of an Ab are essential for binding to its Ag. In fact, the Ag-binding site of an Ab usually involves only 20–33% of the residues [25]. These residues have been designated as specificity determining residues (SDRs) [26]. Therefore, a murine Ab can be humanized through grafting only restricted to its SDRs onto the human template, minimizing the immunogenicity [27].

Another strategy of humanization, termed resurfacing, was proposed by Padlan (1991) and involves the replacement of solvent exposed murine framework residues in the variable regions with human residues [28–30]. Finally, 'Guided selection' is a process that transfers the specificity of a murine mAb to novel human mAbs by creating a hybrid library of the murine heavy chain and random human light chains, then the selecting for binding antibodies and repeating the process with the human light chain isolated and a library of human heavy chains. Adalimumab (Humira®)

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Table 1
FDA-approved recombinant antibodies by august 2005

Year	Product Name	Target	Product type	Technology	Indication	Developer	DCI
1986	Orthoclone OKT®3	CD3	Murine IgG2a	Hybridoma	Transplant rejection	Ortho Biotech	muromonab-CD3
1994	Reopro®	GpIIb/gpIIIa	Chimeric Fab		Cardiovascular disease	Centocor/Eli Lilly	abciximab
1997	Rituxan®	CD20	Chimeric		Non-Hodgkin lymphoma	Biogen-IDEC	rituximab
1997	Zenapax®	CD25	Humanized IgG1		Transplant rejection	Protein Design Labs	daclizumab
1998	Remicade®	TNF-α	Chimeric IgG1		Crohn disease Rheumatoid arthritis	Centocor	infliximab
1998	Simulect®	CD25	Chimeric IgG1		Transplant rejection	Novartis	basiliximab
1998	Synagis®	RSV	Humanized IgG1		Respiratory syncytial virus	Medimmune	pavilizumab
1998	Herceptin®	Her-2	Humanized IgG1		Breast cancer	Genentech	trastuzumab
2000	Mylotarg®	CD33	Humanized IgG4 toxic conjugate		Acute myeloid leukaemia	Wyeth	gemtuzumab
2001	Campath®-1H	CD52	Humanized IgG1	Reshaping	B-cell chronic lymphocytic leukaemia	ILEX Pharmaceuticals	alemtuzumab
2002	Zevalin®	CD20	Murine		Non-Hodgkin lymphoma	Biogen-IDEC	ibritumomab tiuxetan
2002	Humira®	TNF-α	Human IgG1	Phage display guided selection	Rheumatoid arthritis	Abott	adalimumab
2003	Bexxar	CD20	Murine-iodine conjugate		B cell non-Hodgkin's lymphoma	Corixa	tositumomab
2003	Xolair	IgE	Humanized IgG1	Phage display	Asthma, allergy	Genentech	omalizumab
2003	Raptiva®	CD11a	Humanized IgG1		Psoriasis	Genentech	efalizumab
2004	Erbitux	EGFR	Chimeric IgG1		colorectal cancer	ImClone	cetuximab
2004	Avastatin	VEGF	Humanized IgG1	CDR grafting directed mutagenesis FR	colorectal, breast, lung cancer	Genentech	bevacizumab
2005	Tysabri	CD40	humanized IgG4k		Multiple sclerosis	Elan /Biogen	natalizumab

Abreviations: TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor, EGFR: epidermal growth factor receptor, RSV: respiratory syncytial virus.

is the first phage-display-derived human antibody and was generated by "guided selection" starting from a murine monoclonal antibody [31,32].

Despite the reduction of proportion of murine sequences, the level of the immunogenicity of humanized Abs range from negligible to intolerable. The humanized anti-HER2/neu Ab Herceptin(gave as low as 0.1% of HAHA in breast cancer patient [33] while the humanized A33 Ab elicit 49% of HAHA among colon cancer patients treated with this Ab [34]. This immunogenicity might result from human anti-human antibody (HAHA) responses, in particular to the idiotype [35].

It will be interesting to observe the immunogenicity of fully human antibodies in therapeutic settings. To date, it is difficult to determine whether "fully human" mAbs are less immunogenic than humanized mAb as full immunogenicity data are available for only one mAb developed from phage-displayed libraries (Humira®).

Finally, anti-allotype reactions are predicted to occur during therapy of a genetically diverse population with a single antibody reagent.

2.3. Deimmunisation

As an alternative, Biovation (UK, www.biovation.co.uk) has developed the DeImmunisation(technology consisting of identification and removal of T helper (Th) cell epitopes from antibody [16,17]. Two products of DeImmunisation(are actually under clinical trials: J591, a modified antibody binding to prostate specific membrane antigen (PSMA) and the radiolabelled antibody Tromboview.

2.4. Primatized Abs

Another method for obtaining antibodies that are closely related to human Ab involves the use of non-human primates. Primate antibodies are more similar in sequence to human antibodies than are murine antibodies and are less susceptible to be immunogenic in humans. Indeed, the gene segments of macaques are as closely related to human immunoglobulin genes than human genes to each other [36,37].

Three primatized Abs directed against human antigens [IDEC C9.1, IDEC 114 and IDEC 151] are cur-

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Table 2
Immunogenicity of engineered antibodies

AAR	Murine Ab	Chimeric Ab	Humarized Ab
Marked	84%	40%	9%
Tolerable	7%	27%	36%
Negligible	9%	33%	55%

According to Hwang et al. [214], Ab's immunogenicity was classified as 'negligible' if detectable in less than 2% of patients, as 'tolerable' if detectable in 2–15% of patients and as 'marked' if present in more than 15% of patients.
Abbreviations: AAR: anti-antibody response, Ab: antibodies.

rently undergoing clinical trials (Biogen Idec Inc., Cambridge, MA) and preliminary results do not show any anti-primate antibody response [38]. In addition, Biogen Idec is developing lumiliximab, a primatized anti-CD23 macaque/human chimeric antibody that inhibits the production of the IgE antibody, for the potential treatment of allergic conditions [39].

3. Fully human antibodies

Monoclonal antibodies of human origin may have greater therapeutic value, thus several methods have been developed to generate human mAb (Humab): selection from human hybridomas, from transgenic mice or from *in vitro* libraries.

3.1. Human B cell hybridoma

The recovery of stable human B cell hybridoma producing high-affinity IgG mAbs is rarely achieved, lacking of a suitable human myeloma cell line. The best results are obtained using heteromyelomas (mouse x human hybrid myelomas) as fusion partners. However, the mouse-human heteromyelomas that have been used for fusion with human lymphocytes are often unstable. As an alternative, human antibody secreting cells can be immortalized by Epstein-Barr virus (EBV) infection. However, EBV-infected cells are somewhat difficult to clone, and usually produce only very low yields of immunoglobulin [40]. Recently, an improved method of B cell immortalization by EBV involving the addition of a polyclonal B cell activator (CpG) [41] has been described.

The final argument restricting the human B cell hybridoma approach is that human circulating antibody repertoire does not generally contain specificity to "self" proteins, which represent the majority of target for human antibody therapeutics.

3.2. Mice producing human antibodies

3.2.1. Humanized SCID and TRIMERA mice

Another approach for generating human mAbs consist of transplanting a functional human immune system into immunodeficient mouse strains, such as Severe combined immunodeficient (SCID), SCID-bg, Trimera or $\gamma c^{-/-}$/RAG2$^{-/-}$ mice.

Severe combined immunodeficient (SCID) mice, lacking mature T and B cells and virtually devoid of endogenous serum immunoglobulins, can be successfully reconstituted with human peripheral blood lymphocytes (PBLs). Such mice reconstituted with a competent human immune system would represent an invaluable tool for producing large amounts of human immunoglobulins, after immunization with antigen. However, the use of SCID mouse can be limited by shortened lifespans, spontaneous production of functional lymphocytes with ageing, and residual innate immunity leading to variable levels of engraftment. Natural killer (NK) functions in particular would be detrimental to engraftment of human lymphoid cells [42]. For example, depletion of NK activity in SCID-bg mice facilitated engraftment of human PBL from anthrax-vaccinated (AVA) donors. Stable recombinant cell lines producing human monoclonal antibodies were generated by hybridoma formation and human anti-PA neutralizing mAb of high affinity were obtained [43]. Other immuno-deficient strains of mice were developed, for instance the $\gamma c^{-/-}$/RAG2$^{-/-}$ mice. In addition to T- and B-lymphocyte deficiency, $\gamma c^{-/-}$/RAG2$^{-/-}$ mice are completely deficient in NK activity [42].

Alternatively, the Trimera mouse has been developed through a three-step process [44]. Firstly, a normal mouse host is rendered immuno-incompetent by a lethal split-dose total body irradiation. Secondly, the myeloid and erythroid lineages are reconstituted by transplantation of bone marrow cells from a genetically immune-deficient mouse donor. Thirdly, the resulting preconditioned mouse is transplanted with human cells that can be maintained in the host. Even if Trimera mouse was considered as a potential source of monoclonal antibodies, its use has been, so far, restricted to a model for investigation of human diseases.

3.2.2. Transgenic mice

The availability of transgenic strains of mice expressing human Ig genes (XenoMouse®, HuMab^{TM} Mouse, Transchromo Mouse) provides an alternative method for isolating human mAbs [45]. Rearrangement and hypermutation occurrence was an indication

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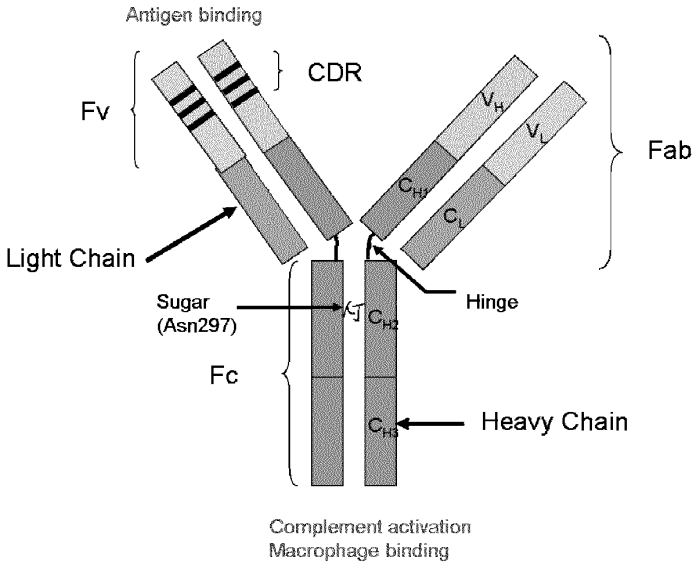


Fig. 1. Schematic representation of an immunoglobulin G (IgG). An IgG is composed of two identical light chains (each composed of two domains, labelled V_L and C_L and two identical heavy chains (each composed of four domains, labelled V_H, C_{H1}, C_{H2} and C_{H3}). Antigen-binding surface is formed by variable domains of the heavy (V_H) and light (V_L) chains. Effector functions, such as complement activation and binding to cytotoxic cells, are mediated by the Fc region.

that the endogenous cell signaling machinery of mice was compatible with human immunoglobulin sequence elements [46] and would constitute a marked advantage.

XenoMouse® strains, developed by Abgenix (Fremont, CA), were engineered by functionally inactivating the murine heavy chain and κ light chain Ig loci and incorporating megabase-sized inserts of human DNA (YAC) carrying Ig heavy chain and κ light chain loci that express the vast majority of the human Ab repertoire [47,48]. Three different strains of XenoMouse® mice have been produced, constrained to class switch from IgM to IgG1, IgG2 or IgG4. Recently, the immune repertoire of XenoMouse® strains has been increased by the introduction of the entire human Igλ locus [46]. To date, results obtained in preclinical and early clinical trials with human antibody from Xenomouse® mice confirm their lack of immunogenicity [49,50]. However, human antibodies from mice can be distinguished from human antibodies produced in human cells by their state of glycosylation, particularly with respect to their Galα1-3Gal residue [51]. This carbohydrate residue is widely distributed among non-primate mammals. Anti-Gal antibodies are produced in humans throughout life, as approximately 1% of circulating Ig [52]. Thus, antibodies bearing that residue would probably be subject to rapid immune clearance [53].

As an alternative strategy, human minichromosomes (derived from human chromosome 2 and 14) containing the complete germline clusters for heavy and κ light chains were introduced into mice, called Transchromo mice [54,55]. These mice, developed by the Kirin Brewery Company (Japan), are capable of producing every subtype of fully human Ig, including IgA and IgM. However, the instability of the transchromosome carrying the Igκ locus was particularly detrimental, as hybridoma production was less than 1% of that seen in wild type mice. An instant solution to this problem was to cross-breed the Kirin TC mouse carrying the human chromosome fragment 14 (locus IgH), with the Medarex YAC transgenic mouse carrying about 50% of the Igκ locus. The resulting mouse (KM) performed as well as normal mice with regard to immune responsiveness and efficiency of hybridoma product [55].

3.2.3. *Antibody display*

An approach considered as a breakthrough for producing fully human antibody is the selection from antibody gene repertoires expressed either *in vivo* on the surface of cells or filamentous bacteriophages [56,57], or *in vitro*. These strategies of selection of antibodies from large antibody repertoire depend on the linkage between genotype and phenotype, enabling recovery of the DNA encoding the selected antibody fragment.

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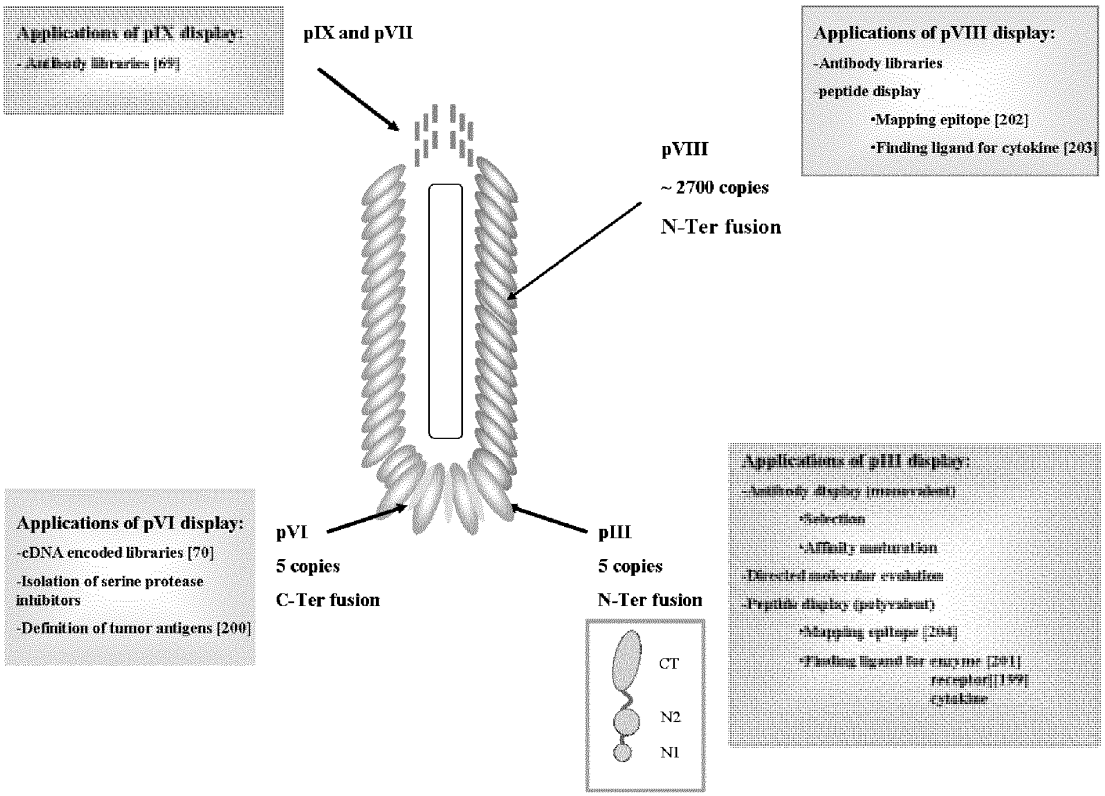


Fig. 2. Illustration of various M13 phage display applications. The large diversity of display formats of peptide or protein has allowed various applications as illustrated in this figure, including selection of high affinity antibodies, directed molecular evolution, screening of cDNA libraries, mapping epitope, and finding ligand for cytokine, enzyme or receptor.

3.2.3.1. Phage display

This strategy is currently the most widely used and well-established technique for antibody display and library screening [58]. Large repertoires of scFv or Fab antibody genes are cloned into phagemid vectors as a fusion product to one of the phage coat protein genes. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody display on the phage surface. During the screening procedure generally termed "biopanning", phages that display a relevant antibody will be retained on a surface coated with antigen while non-specific phages will be washed away. Multiple rounds of panning are made possible and necessary to select the best binders.

Naïve, Immune and synthetic repertoires

The different types of antibody libraries are distinguished by the source of their repertoires: naïve, immune or synthetic. Naïve libraries are constructed by cloning the antibody variable domain genes from non-immunized donors [59,60]. Since the description of the first antibody libraries, many efforts have been done towards the obtaining of universal large sized repertoires. The theoretical justification for large repertoires was that it should be possible to isolate, from a large repertoire (10^{10}–10^{11}), antibodies with nanomolar affinities against any antigen [61,62]. The key factor limiting the obtaining of very large repertoires is the bacterial transformation step, inherent to the library construction. Even if techniques based on so called 'combinatorial infection' [63,64] have provided an elegant means of breaking this technological barrier, the vast majority of, so far described, 'naïve' repertoires, with a complexity over 10^{10} clones, have been assembled through the massive and tedious accumulation of small-sized sub-repertoires [60].

As a definition the complexity of a given library is the overall number of different VH–VL combinations obtained at the DNA level. But the experience has shown that there is a significant difference between the encoded diversity and the displayed diversity for differ-

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ent reasons such as toxicity upon bacterial expression, non-proper folding or assembly, competition between wild type and antibody-linked gene III molecules, proteolysis of the displayed moiety, and many others.

Lastly, it became obvious that the major problem was not the assembly of a large repertoire but rather its maintenance over time and the retardation of the ineluctable drift of its content. This clearly explains that the current tendency is rather focused on smaller or biased repertoires obtained from immunized subjects [60]. An immune library has two main characteristics: it will be enriched in antigen-specific antibodies and some of these antibodies will have undergone affinity maturation by the immune system.

Nevertheless, some examples of universal large sized synthetic [62,65] and semi-synthetic [66] repertoires are available. They are based on the use of a limited number of universal frameworks selected for their capacity to be over-expressed in *E. coli*. According to the technique employed, CDRs are totally synthetic or derived from a pool of naturally expressed ones.

Display formats and optimization

Display method have been developed based on fusion to the C-terminus of the λ phage tail protein pV, or both the N- and C-terminus of the capsid D protein, which is part of the phage head. Further double stranded DNA phage display systems have been described using bacteriophages T4 [67] or T7.

The filamentous bacteriophage M13 has been by far the platform of choice for antibody display (Fig. 2). Although M13 coat protein pIII and pVIII [68] display formats have been the most instrumental in the construction of antibody libraries, alternative phage display systems have been reported, like those based on the minor coat protein pIX [69] or pVI [70]. Because the success of phage display depends not only on the diversity of the library at the DNA level, but also on the efficiency with which the encoded proteins are displayed on the phage surface, extensive studies of M13 assembly and structure have enabled improvements in phage display technology [71]. Indeed, phage selections often suffer from the amplification of non-specific binding molecules. In order to alleviate this problem, three groups have developed a system coupling the binding of a displayed peptide or protein to its target with the amplification of the displaying phage: selectively infective phage (SIP) [72], selection and amplification of phage (SAP) [73], and direct interaction rescue (DIRE) [74]. The 'selectively infective phage' technology (SIP) exploit the modular structure of the phage protein pIII, which consists of three domains: N1, N2 and CT. The N-terminal N1 domain is absolutely essential for *E. coli* infection, while the CT domain is absolutely essential for phage morphogenesis. [75]. Thus, SIP consists of two components: (i) a phage particle made non-infective by replacing its N-terminal domains of pIII with an antibody fragment and (ii) an "adaptater" molecule in which the antigen is linked to N-terminal domains. Infectivity is restored when the displayed protein binds to the ligand. Consequently, phage propagation is strictly dependent on the protein-ligand interaction [76].

A vector system allowing for the display of bivalent Fabs fused to leucine zippers on phagemid virions has been reported by Lee et al. [77]. The "bivalent display" format is a way to effectively mimicking the binding avidity of natural antibodies and greatly reducing the off-rate for phage bound to immobilized antigen.

3.2.3.2. Yeast Display, bacterial display, Anchored periplasmic expression (APEX)

A complementary approach utilizes the display of antibody libraries on the surface of bacteria [78] or yeast cells, most commonly *Escherichia coli* and *Saccharomyces cerevisiae*. The display of Ab on the surface of bacteria is not only an alternative expression system for the screening of binders from libraries, but opens new potential applications, like the delivery of passive immunity to mucosal body surfaces [79] or whole cell catalyst [80]. Unlike phage, the relatively large size of bacteria and yeast allows screening by flow cytometry [81].

Recently, a protein library-screening technology based on anchored periplasmic expression (APEX) has been developed [82]. In this method, proteins are anchored on the periplasmic face of the inner membrane of *E. coli*. After disruption of the outer membrane by tris-EDTA-lysozyme, the inner membrane-anchored proteins bind fluorescent ligands, allowing screening by flow cytometry.

Baculovirus displaying antibody moieties such as scFv fragments have been constructed for targeting specific cell types in the gene therapy field. But as for today the baculovirus system was not considered as an attractive choice for the construction of antibody libraries [83].

3.2.3.3. In vitro display technologies

One limitation of the *in vivo* selection systems is the library size that could be generated. The efficiency of transfer of DNA into cells often limits the library size to 10^9–10^{10} members. In addition, selection in the

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Table 3
Recombinant antibody fragments

Ab fragments	Composition	Features	Properties	Applications
dAb	V_H or V_L	PM: 11-15 kDa • Llama single domain Ab fragment (VHH) [114] •NAR [117-118]	Expression at high level in microbial culture Solubility, temperature stability, high stability under denaturing conditions that far exceed those of IgG, Fab or scFv [10]	• *In vitro* selection (phage display) and affinity maturation • Can be formatted into larger molecules to create drugs with prolonged serum half-life • Immunocytochemical, immunohistochemical, immunoblot analysis [205] • Intrabodies
Fv	V_HV_L	PM: 25-30 kDa	Rapid blood clearance Better tumor penetration	
scFv	V_HV_L	Stabilized by a linker $(Gly_4Ser)_3$ [211]	More stable or higher affinity for the antigen	
dsFv	V_HV_L	Stabilized by disulphide bridges [93]		
Fab	$(V_{H1}+C_{H1})$ (V_L+C_L)	PM:50 kDa	Better stability than scfv Prolonged circulating half life *in vivo*	Immunotherapy (eg Reopro®)
Fab-PEG			Prolonged circulating half-life Reduced immunogenicity Increased solubility and resistance to proteolysis [105]	Immunotherapy
Diabody Triabody Tetrabody		Reducing the linker length results in the formation of dimers, trimers or tetramers [90]	Incresased avidity high functional affinity	
$(scFv)_2$	scFv-scFv	(scFv)2 produced using disulphide bridges or by joining the two scFv fragments with a third polpypeptide linker	• Cross-linking tumor and effector cells ⇨	• Redirect cytotoxic immune effector cells for enhanced killing of tumor cells by ADCC [207]
scDiabody Tandem diabody		The formation of either a scDiabody or a tandem diabody depends on length and aminoacid composition of the linkers [206]	Drawbacks: Short half-life, Poor accessibility to tumor sites	• Enrich the tumor/normal tissue localization ratio of chemo or radiotherapeutic agents [215]
Di-Minibody		scFv fused with amphipathic helixes [100], immunoglobulin CH3 domains [101], a 'knobs into holes' device, a barnase-barstar couple [103]		• Target two tumor-associated antigens for down-regulation of multiple distinct cell proliferation/survival pathways [208]

context of the host environment (*E. coli*) could lead the loss of potential candidates due to their growth disadvantage or even toxicity for *E. coli*. The most popular *in vitro* display technologies are ribosome display [84] and mRNA display [85] based on an original idea from G. Kawasaki (US patent n° 5,643,768 and US patent n° 5,658,754). These *in vitro*-based antibody selection methods have proven to be successful in the construction and selection of libraries with a high diversity and complexities (potentially up to 10^{14} members). Inherent characteristics of the *in vitro* systems could obviously be turned into advantages:

- More easily amenable to an automated process,
- The RT-PCR step between screening rounds can be performed according to error prone conditions, thus generating an additional degree of diversity [86,87].
- An *In vitro* process is more likely to tolerate screenings under non-physiological conditions such as elevated temperature or a highly denaturant environment [87].

Other potential *in vitro* systems for antibody display are covalent antibody display (CAD) and polysome display [88]. In the covalent display technology, a protein is fused to P2A, a bacteriophage DNA-nicking protein that covalently binds its own DNA and thereby be subjected to selection regimes similar to those for phage display [89]. The polysome display is a modified ribosome display method, by exploiting the interaction between a tandemly fused MS2 coat-protein (MSp) dimer and the RNA sequence of the corresponding specific binding motif, C-variant [88].

4. Antibody design (Table 3)

4.1. Antibody fragments

For many therapeutic applications, the antibody activity (as cytokine inactivation, receptor blockade or

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Table 3, continued

Ab fragments	Composition	Features	Properties	Applications
Tetra-Minibody	• MoaFv [95]: the $CH1$ and CL domains of the Fab fragment replaced with heterotetrameric molybdopterin synthase (MPTS). • scFv-streptavidin: fusion of scFv with streptavidin [209] • Fusion with the tetramerization domain of p53 [216]			
Immunotoxins	Plant toxins: ricin A chain [213], saporin Bacterial toxins: Pseudomonas exotoxin [212], cholera toxin	Potential immunogenicity of non-human-derived toxins		Tumor cells killing [104] (eg Mylotarg®)
Ab-drug	Doxorubicin Calicheamicin	Potential lost of antibody reactivity upon conjugation		Targeted delivery of drugs to a tumor site
Ab-radionuclide	Yttrium-90 (ex: Zevalin®) Iodine-131 (ex: Bexxar®)			Immunoscintigraphy Radio-immunotherapy (RIT)
Immunocytokines	A variety of cytokines (IL-2, IL-12) linked to full length Ab or scFv fragment [4]			Activation of anti-tumoral immune response
Ab-enzyme	DNase, RNase [110]	Immunogenicity?		Antibody directed enzyme prodrug therapy (ADEPT) [105-111]
Peptibodies	Fusion of a peptide, capable of binding Ab effector molecules, to scFv or Fab [125]	Easy production / full-size Abs		Recruitment of effector's functions
Trovobodies	T-cell epitopes inserted into loops of the $CH1$ domain of APC-specific antibodies [133-140]			Delivery of T cell epitopes to antigen presenting cell (APC)

Monospecific immunoglobulin fragments are represented in white, bispecific antibodies are indicated in light grey and conjugated antibodies are shown in dark grey.

viral neutralization) does not require the recruitment of effector functions through the Fc portion. According to the therapeutic application, all of Ab's features should be taken into consideration: size, tissue penetration, distribution, half-life, effector functions, affinity, stability and immunogenicity. This can explain that smallest Ab (scFv or Fab) fragments may be preferred. Consequently, one of the tendencies of antibody engineering has been to reduce the size towards a minimum antibody fragment, retaining both binding affinity and specificity. In addition, smallest Ab fragment can be functionally expressed in *Escherichia coli* and yeast, which would dramatically reduce the cost associated with large-scale mammalian cell culture.

The Fv fragment, formed by the heterodimeric association of the two variable domains V_L and V_H of the light (L) and heavy (H) chain, respectively, can be genetically engineered into single chain Fv fragment (scFv) with a flexible polypeptide linker. The most commonly used linker is a flexible decapentapeptide (Gly4Ser)3. Changing the linker length between V-domains was capable of inducing oligomerization into diabodies, or even into higher order valency antibody fragments (triabodies, tetrabodies), potentially increasing the avidity [90]. Diabodies, the most characterized of these molecules, have shown high functional affinity, greater tumour retention and slower systemic clearance than their monovalent counterparts in pre-clinical studies [91]. To stabilize the association of the V_H and V_L domains, different linkage strategies have been proposed, for instance through disulfide bridges [92,93] and 'knock into holes' mutations [94]. An alternative format to scFv, named MoaFv, has been proposed to stabilize the Fv fragment and restore the bivalency of the antibody [95]. This MoaFv was constructed by replacing the C_{H1} and C_L domains of the Fab fragment with heterotetrameric molybdopterin synthase (MPTS).

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The fragment antigen binding (Fab) is a heterodimer of V_H-C_{H1} and V_L-C_L linked together through a disulfide bond. In comparison with whole antibodies, small antibody fragments such as Fab or scFv exhibit better pharmacokinetics for tissue penetration. However, Fab and scFv are monovalent and often exhibit fast off-rates and poor retention time on the target.

The half-life of circulating antibody fragment (Fab, scFv) can be improved by site-specific coupling of polyethylene glycol (PEG) to the fragment, so called PEGylation [96]. For example, site-specific pegylation into the hinge region has prolonged the circulating half-life of an anti-TNF-α human fragment (CDP 870, Celltech) to 14 days. Covalent attachment of PEG increases the circulating *in vivo* half life of the antibody fragment, increasing the apparent size of the antibody fragment above the glomerular filtration limit. PEG may have the additional benefit of increasing solubility and resistance to proteolysis, and almost reducing immunogenicity [97]. Kitamura et al. have shown that PEG attachment to an intact murine monoclonal antibody reduced the HAMA response significantly when compared to the parent intact antibody [98].

4.2. Bispecific antibodies

Bispecific antibodies (BsAb) bind to two different epitopes and have been used to crosslink various cells and molecules. Various methods for generating bispecific antibodies have been developed: generation of hybrid-hybridoma or quadroma [99], chemical and genetic coupling of Fab fragment. Multimeric Ab fragments may be obtained by linking them to molecules which tend to self-associate, such as amphipathic helices [100], coiled-coil structures, immunoglobulin constant domains [101] and molecules which are homomultimeric. Recently, a new format of BsAb has been proposed, consisting of two scFv, one for human epidermal growth factor receptor 2 (HER2)/neu and the other for CD16, heterodimerized by a 'knobs-into-holes' device from the C_{H3} domains of the human IgG1 Fc fragment [102]. Another multimerisation system, based on the interaction between the prokaryotic ribonuclease Barnase (110 aa) and its inhibitor Barstar (89 aa) has revealed important advantages [103]. The use of such constructs is based on the exceptionally high association constant (up to 10^{14} M^{-1}) of the barnase-barstar couple.

4.3. Conjugated antibodies or "armed" antibodies

Ab fragments have been fused with a vast range of molecules to introduce different functionalities including radionuclides, toxins, or drugs for cancer cell killing, enzymes for prodrug therapy and cytokines to stimulate the anti-tumor immune response.

4.3.1. Tumor cells killing

For example, monoclonal antibodies directed to tumour-associated antigens or antigens differentially expressed on the tumor vasculature have been covalently linked to a drug such as calicheamicin (Mylotarg®, Wyeth) or a radionuclide (Zevalin®). These antibodies have been designed to deliver their toxic load directly to cancer cells, avoiding the toxicity associated with systemic administration. Imunotoxins are composed of Fv fragments of antibodies that bind to cancer cells fused to a truncated form of a very potent bacterial toxin (e.g. *Pseudomonas* exotoxin, ricin A chain). The antibody moiety specifically directs the toxin to cancer cells [104].

4.3.2. Activation of anti-tumoral immune response

Similarly, cytokine-antibody fusions (e.g. TNF-α, IFN-γ IL-12 or IL-2) have shown to initiate tumour regression in several systems [4]. These immunocytokines allow the local activation of the anti-tumoral immune response, avoiding the dilution effect associated with systemic cytokine administration. Another category of antibodies with high specificity and affinity can be developed to bind specific cytokines or their receptors, inhibiting the detrimental effect of the cytokine [3].

4.3.3. Antibody-directed enzyme prodrug therapy (ADEPT)

Enzyme may also be conjugated to antibodies in antibody-directed enzyme-prodrug therapy [105]. ADEPT involves the pre-targeting of prodrugs to tumors. An Ab-enzyme fusion protein is first administered and allows localizing to the tumor, followed by the administration of the prodrug which is activated by the enzyme at the tumor site [106]. This approach has been recently applied to various tumour therapies [107–109]. For example, a dimeric immunoenzyme comprising an anti-CD22 diabody and *Rana pipiens* liver ribonuclease was recently generated [110]. Previous clinical trials have shown evidence of tumor response; however, the activated drug had a long half-life, which resulted in dose-limiting myelosuppression. Also, the

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targeting system, although giving high tumour to blood ratios of antibody conjugate (10 000: 1) required administration of a clearing antibody in addition to the antibody-enzyme conjugate [111].

4.4. Alternative immune repertoire [112]

Full-length antibodies, Fab and scFv fragments provide an antigen-binding surface comprising six CDR loops. Some target molecules are refractory to the immune repertoire, particularly those with cavities. The specific recognition of these target molecules would require a small penetrating loop for tight binding. The natural mammalian antibody repertoire does not encode penetrating loops and only rarely has the propensity to select this type of antibody [113]. In camelid species (camels, dromedaries, llamas), a significant proportion of the serum immunoglobulins is constituted by antibodies naturally devoid of light chains, called heavy-chain antibodies or HCAbs [114]. HCAbs lack the first domain C_{H1} but harbour an intact variable (V) domain (VHH) encoded by different V genes. This HCAb has been found to be physically very stable and is produced at very high levels in *Escherichia coli*. In addition, the CDR3 (up to 24 residues in llamas) is unusually long and such CDRs are especially suited for binding to the inner part of cavities [115].

On the basis of theses observations, Domantis (www.domantis.com) has developed a series of large and highly functional libraries of fully human V_H and V_L domain antibodies (dAbs). These dAbs correspond to the variable regions of either the heavy (V_H) or light (V_L) chains of human antibodies.

More recently, the immunoglobulin isotype novel antigen receptor (IgNAR) has been discovered in the serum of the nurse shark (*Ginglymostoma cirratum*) as a homodimeric heavy-chain complex, which also naturally lacks light chains. NARs are composed of five constant domains and a single variable domain with two CDR-like regions [116,117]. The variable domains of these unique molecules are candidates for the smallest antibody-based immune recognition units. A library of V(NAR) domains with extensive variability in the CDR1 and CDR3 loops displayed on the surface of bacteriophage has been produced. The content of this library was further validated through selection against the Apical Membrane Antigen-1 (AMA1) from *Plasmodium falciparum*. In addition, a recombinant V(NAR)s with nanomolar affinities for AMA1 could be produced at good yields in *E. coli*. [118].

4.4.1. Alternative scaffolds

4.4.1.1. Ankyrin repeat protein

Structural characteristics of ankyrin repeat (AR) protein incited to exploit AR domain as a scaffold for the construction of libraries of novel binding molecules [119]. Binz et al. have designed an AR module composed of fixed framework positions and randomized potential interaction positions [120]. These proteins are more amenable to be expressed in large amount; they are generally soluble, monomeric, devoid of disulfide bonds and stable under physiological conditions. Recently, the potential of AR protein has been demonstrated as intracellular kinase inhibitors [121].

4.4.1.2. Anticalin and duocalin

Lipocalins have also been recruted as scaffold for the design of artificial binding proteins termed Anticalin® (Pieris proteolab AG) [122,123]. Compared with antibodies, lipocalins, which exhibit biotechnological advantages due to their smaller size, are composed of a single polypeptide chain and they exhibit a simpler set of four hypervariable loops that can be more easily manipulated at the genetic level. Schlehuber and Skerra have also reported the construction of a functional fusion protein from two independent anticalins, a so-called duocalin [124].

4.5. Pepbodies

Pepbodies are obtained by fusion of a peptide, capable of binding one or several Ab effector molecules, to scFv or Fab fragment. These targeting fragments are able to efficiently, recruit some of the effector's functions, normally associated with the Fc fragment, such as complement activation [125]. Such pepbodies have been constructed, by introducing C1q-binding peptides into a loop of a C region domain. These pepbodies have retained the ability to bind both the antigen and C1q.

4.6. Intrabodies and transbodies

4.6.1. Intrabodies [126,127]

Intracellular Abs, also called intrabodies, are usually scFv [128] or dAbs [129], expressed within the confines of the intracellular environment. During the past decade, there has been growing interest in the use of such Ab fragment, in therapy of human diseases [130], targeting intracellular molecules such as tumour antigens and in functional genomics by blocking or modulating the activity of proteins or protein domains. Intra-

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bodies can also be designed to modulate the function of its target molecule in other ways. For example, it is possible to design intrabodies to relocate its target molecule to another subcellular location.

However, the cytoplasmic expression of scFvs is generally limited by their instability and insolubility. The reducing environment of the cytoplasm prevents the formation of the intra-chain disulfide bridges [131], which are critical in folding almost all antibodies. Consequently, most Abs does not fold properly and are inactive inside cells. A number of approaches have been devised to overcome the limitation imposed by the cellular environment.

To overcome this problem, Auf der Maur et al. have proposed the "quality control" system that allows for a rapid selection of stable and soluble antibody frameworks suitable for intracellular applications [131]. This approach consists of constructing randomized hypervariable loop libraries on scFv frameworks that have been pre-selected for their intrinsic high stability and solubility in an intracellular environment.

Intracellular antibody capture (IAC) technology [132] is an approach based on selecting intrabodies starting from diverse scFv phage antibody libraries, which are initially screened against an antigen *in vitro*, and subsequently screening the selected scFv in a yeast *in vivo* antibody-antigen interaction assay.

4.6.2. Therapeutic antibody gene transfer

A tentative answer to overcome some issues in the domain of passive immunotherapy has been proposed by Bakker et al. [133]. The approach, taking advantage of the existing gene therapy protocols, is the possibility of raising therapeutic antibodies after DNA transfer *in vivo*. Genes encoding fully human antibodies targeting pathogens might be complementary to existing treatments and help controlling infectious diseases.

4.6.3. Transbodies

Recently, Heng and Cao have proposed to test the possibility of fusing protein transduction domain (PTD) to scFv antibodies [134]. PTD are short peptide sequences that enable proteins to translocate across the cell membrane and be internalized within the cytosol. This would result in a 'cell-permeable' antibody or 'Transbody'. Correct conformation, folding and disulfide bond formation can take place prior to introduction into the target cell. Thus, the process, still at hypothesis stage, has been proposed to possess several advantages over standard intrabody technology and to be potentially more efficient than the well-established gene silencing through RNA interference.

4.6.4. Antibodies and vaccination

4.6.4.1. Idiotypic network

Immunization with anti-idiotype (Id) antibodies represents a novel approach to active immunotherapy [135, 136]. It has been proposed that an anti-idiotypic Ab (anti-anti-X) may resemble the external Ag X and thus carry its 'internal image' [137]. Idiotypic-based vaccines contain neither nominal Ag nor its fragments. This includes the possibility that Id vaccines would be devoid of undesired side or toxic effects, which might sometimes be associated with some candidate antigens. In addition, one of the major problems of human cancer is 'immune tolerance', which might be tentatively broken by an appropriate anti-Id vaccine [136].

4.6.4.2. Antigenized antibodies

Antigenization of Abs, consist of the expression of foreign oligopeptides located into the hypervariable loops of an antibody molecule [138]. Immunization with antigenized antibodies is an alternative method to focus the immune response against defined epitopes of foreign antigens, and a way to deliver a peptide immunogen with an extended half-life. Musselli et al. have demonstrated that the severity of experimental allergic encephalomyelitis can be greatly attenuated by active immunization with antigenized Ab expressing synthetic peptides of the TCR, which are a key regulatory element in this disease [139].

4.6.4.3. Troybodies

Troybodies are APC-specific antibodies with T-cell epitopes inserted and "hidden" into loops of the CH1 domain (Constant region), without disrupting immunoglobulin folding. When they bind to their target APC, the T-cell epitopes are released, by antigen processing, and presented in association with MHC class II molecules to $CD4^+$ T cells. Targeting of antigens to antigen presenting cell (APC) results in an enhanced antigen presentation and T cell activation [125,140].

4.6.5. Catalytic antibodies

Among the multiple facets of the antibody molecule, one of the most intriguing is the capacity of naturally supporting a catalytic activity. A major and still controversial question is whether catalytic or more generally enzymatic activity of some natural antibodies represent function or dysfunction of these molecules [141]. According to the literature, antibody catalytic activities have been often associated to pathogenic situations such as autoimmune diseases. On the other hand, some other works have emphasized the beneficial role of cat-

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alytic antibodies, considered in that context as "defensive enzymes" [142]. Serine protease or other peptidase activities have been reported for naturally occurring catalytic antibodies [143]. Remarkably, in a number of examples the light chain has been precisely characterized as the active component for proteolysis [144].

A wide array of therapeutic applications is conceivable for catalytic antibodies, including anti-viral intervention or pro-drugs activation. In the latter case the major advantage would be the lower immunogenicity of a catalyst perceived just as a natural molecule by the host's immune system. In the domain of treatment for drug addiction, interesting data have been reported concerning catalytic antibodies capable of breaking down cocaine to systemically inert molecules [145,146].

Isolating antibodies acting as powerful catalytic entities is still a great challenge; the reasons underlying that situation are essentially the following:

- Catalytic antibodies are often selected for their capacity to stabilize the transition state of a given chemical reaction, which impose to design, as immunogens, transition state analogues. The major difficulty being to chemically synthesize stable molecules mimicking a highly ephemeral structural entity, and very closely similar to the transition state in terms of 3D structure.
- The other constraint is more related to a capacity of screening against a catalytic activity, which could be very different from a standard selection based on affinity for the target.

A comprehensive overview of various strategies to overcome these issues was recently published [147, 148].

5. Expression systems

Clinical applications of antibodies often require large amount of highly purified molecules. The development of very efficient expression systems is essential to the full exploitation of the antibody potential. The expression of functional, correctly folded antibodies or antibody fragments and its scale up to commercial level is a major goal in therapeutic antibodies development. The choice of the most suitable expression system is function of the Ab format (whole antibody, monovalent or bivalent fragment), the application of the Ab (therapeutic, diagnostic, experimental tools), but factors such as scale-up, total annual production, post translational modification and regulatory issues comes also into play. While Bacteria and yeast are suitable for the production of antibody fragments, only mammalian cells or transgenic organisms can be the source of whole antibodies. The huge demand for large amounts of monoclonal antibodies is currently driving the improvement of existing expression systems or the quest for alternative production means.

5.1. Prokaryotic systems

Bacterial systems are suitable for the expression of scFv and Fab, intracellularly or within the periplasm.

5.1.1. E. coli [149]

Efficient recovery of properly folded and active Ab fragment in *E. coli* is strongly primary sequence dependent. Expression of recombinant Ab is typically achieved by fusion to N-terminal signal peptides, which target the protein to the periplasmic space of *E. coli* where chaperones (Skp, FkpA, DsbA and DsbC) assist the folding of the Ig domains and form the correct disulfide bridges to stabilize the structure. However, during accumulation of recombinant proteins in the periplasm of *E. coli*, undesired proteolytic degradations can occur. *E. coli* host strains lacking the major periplasmic proteases (DegP and Prc) have been shown to increase the yield of Fab produced in the periplasm two to three fold [150]. The yield of functional antibody fragments expressed in the bacterial periplasm depends also on the amino acid sequence, being highly variable from antibody to antibody. Alternatively, a correctly folded Fab has been produced in the cytoplasm of *E. coli* trx B gor potential of the cytoplasm is comparable to that of the mammalian endoplasmic reticulum. The expression has been improved via the co-expression of molecular chaperones such as skp [151]. An alternative to the periplasmic and cytoplasmic expression of Abs is their secretion to culture supernatants using the α-hemolysin (HlyA) system of *E. coli* [152].

Until recently it was admitted that the expression of full-length antibodies in the periplasmic domain of *E. coli* was not a reasonably achievable goal. Nevertheless, the assumption was objected by Simmons et al. (2002) who described the rapid and efficient expression of complete IgGs in the periplasm of *E. coli* [153]. The binding properties, of the recovered antibodies, were fully retained while, as expected, the recruitment of the effector functions linked to the Fc domain was lost.

The interesting question of a potential glycosylation, in *Escherichia coli*, was addressed in a work done by Wacker et al. [154]. They have established that the N-

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linked glycosylation pathway found in the bacterium *Campylobacter jejuni* could be transferred into *E. coli*. Although structural differences exist between bacteria and eukaryotic cells, this raises the possibility of expressing glycosylated antibodies or antibody fragments in *E. coli*.

One can imagine that a combination of the two previously described technologies might open up the way to the production of full-length glycosylated antibodies or other complex molecules in *E. coli*. As exciting as could be such a perspective we should not underestimate both, the difficulty of transferring complete glycosylation pathways into prokaryotic hosts and the limitation of expressing large complex molecules into the periplasm of gram negative bacteria.

5.1.2. Alternative prokaryotic systems

Several gram positive bacteria have been used for expressing antibody fragment such as *Bacillus subtilis* [155], *Lactobacilus zeae* [156], *Streptomyces lividans* [157], and *Staphylococcus carnosus* [158].

As the assembly and glycosylation of full-length antibodies is not possible in bacterial systems, mammalian cell culture or transgenic organisms are more suitable for production of these molecules.

5.2. Baculovirus expression system

The baculovirus expression system has been successfully used for the expression of functionally active antibodies [159,161]. Moreover, cassette baculovirus vectors with human Fc regions have been engineered to allow transfer of specific variable regions selected by phage display and subsequent expression as a complete antibody [160]. A major disadvantage of the baculovirus expression system, however, is the catalytic properties of infectious baculovirus that narrows the window for optimal antibody production (generally 3–5 days).

5.3. Mammalian cell culture

Any human antibody IgG subclass can be generated in mammalian cell culture systems using Chinese hamster ovary (CHO) cells, NSO cells (derived from mouse myeloma) or Human embryo kidney (HEK-293) [162]. Mammalian cell culture has emerged as the method of choice for the production of most monoclonal antibodies currently commercialized, although the cost associated with scaling this system up for mass-production purpose are extremely high. Estimate of the current worldwide capacity available for antibody manufacturing using mammalian cell culture range from 413 000 L [163] to 475 000 L [164]. Morrow estimates that 25–50 new mAb products could be approved in the next five years, requiring 15 000–20 000 L each of new capacity. Also, currently available manufacturing capacities could be overhelmed in the near term (1–2 years).

Although improvements in media composition and host cell engineering have led increase of the productivity of mammalian cells [165], alternative expression systems have to be developed.

5.4. Yeast and filamentous fungi

The ability of these organisms to grow in chemically defined medium in the absence of animal-derived growth factors and to secrete large amounts of recombinant protein have made these hosts a system widely used to produce proteins that cannot be functionally expressed in *E. coli*. because of folding problems or the requirement for glycosylation.

Several antibody fragments, such as single-chain antibodies (scFvs) [166] or Fab [167] have been expressed at high yield in *Pichia pastoris*. The methylotrophic yeast *Pichia pastoris* is almost as easy to genetically manipulate as *Escherichia coli* could be, and has an eukaryotic protein-synthesis pathway [166]. In addition, antibody fragments can be produced in *Pichia* under fully validated conditions, which is essential for the production of therapeutics. Protein expression in Yeast is carried out using defined media, and recombinant proteins can be secreted into the medium, enabling purification from a starting material that does not contain a great number of contaminants.

The filamentous fungi *Aspergillus awamori* [168], *Aspergillus niger* [169] and *Trichoderma reesei* (patent n°WO 92/01797) have also been used to express antibody fragment and full-length IgG.

5.5. Transgenic plants

Antibodies extracted from plants, so-called plantibodies, have particular advantages in term of cost of production, ease of scaling up or down production to meet market demand, and freedom from animal-derived pathogens including prions and viruses [170, 171]. The first functional mAb was expressed in plant in 1989 [172]. Since then, plantibodies have been successfully produced in transgenic tobacco, soybean [173], alfalfa [174] and other plants [175]. Plants have proven to be effective systems for producing func-

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tional therapeutic mAbs capable of conferring passive protection against bacterial and viral pathogens in animals [171,176].

To date, plants offer the only large-scale, commercially viable system for production of secretory IgA (sIgA) [177].

Particle bombardment allows the simultaneous introduction of multiple constructs, thereby expediting the recovery of transgenic lines expressing multimeric antibodies such as secretory immunoglobulin (sIgA) [178]. The recent availability of large amounts of secretory IgA plantibodies opens up a number of novel therapeutic opportunities for disorders of the mucosal immune system. In a human trial, the plant derived recombinant sIgA prevented oral re-colonization by *Streptococcus mutans* [175].

5.6. Transgenic animal-based production systems

The mammary gland has generally been considered the tissue of choice to express valuable recombinant protein since milk is easily collected in large volumes. Targeting the production of recombinant protein pharmaceuticals to the milk of transgenic farm animals (goat, cow) could potentially solve many of the problems associated with either microbial or animal cell expression systems: improperly folding of complex proteins and lacking of adequate post-translational modifications [179]. Several reports describe expression of antibodies in the milk of transgenic animals [180] despite limitations by the relatively long interval from birth to first lactation encountered with domestic livestock, the discontinuous nature of the lactation cycle and substantial material investments required to produce transgenic dairy animals [181].

The transgenic production of antibodies in egg white has been reported to take approximately 18 months [182]. The cost of generating non-purified material from transgenic chikens is calculated at $0.5/g. However, this cost does not account for the production of material under cGMP conditions.

Insect, plant and yeast cells are attractive as hosts for the production of recombinant antibodies as they represent potentially inexpensive and versatile expression systems. The principal limitations to the greater use of these hosts for the generation of pharmaceutical proteins are that the N-glycans generated differ from those of human glycoproteins due to the presence of some host glyco-epitopes on expressed glycoproteins which may elicit immune responses in humans, and poor pharmacological activity *in vivo* due to the rapid clearance from the circulatory system of glycoproteins with non human glycans.

5.7. In vitro system

Cell-free protein synthesis systems have been used as an alternative method of producing proteins from cloned genes. Thus, scFv [183] and Fab [184] have been reported to be synthesized in the *in vitro* systems. However, functional Abs with antigen binding activity are obtained only if disulfide formation and rearrangement is allowed to take place during the translation reaction (for example with addition of protein disulfide isomerase (PDI) or chaperones) [185,186]. Despite a certain potential for scale up, cell-free transcription/translation systems are still at a laboratory scale and within the milligram range, limiting their use as production means for research or screening purposes.

5.8. Glycosylation pattern and expression systems

All the above-mentioned expression systems are not equivalent for the post-translational modifications of antibodies, in particular glycosylation. Although the processing of the initial Glc2Man9Glc Nac2 oligosaccharide to Man8GlcNAc2 in the endoplasmic reticulum shows significant similarities between plants, insects, yeasts and mammals, very different processing events occurs in the golgi compartments [187]. Glycosylation profoundly affects the effector functions of IgG antibodies [53]. In particular, an optimal effector function is dependent upon the correct carbohydrate structure at Asn 297 of the Fc region of the two heavy chains. This oligosaccharide is buried between the CH2 domains, forming extensive contacts with amino acid residues within CH2. The terminal β1,4-galactose residues are in close contact with the protein backbone. It has been suggested that the Gal residues are critical for antibody performance, not only in effector functions but also in correct antibody folding [53]. Differences in glycan usage between human and expression hosts (mammalian cells, insect cells plants, yeasts) have caused some concerns that recombinant mAbs could be immunogenic or even allergenic. Consequently, several groups investigated the possibility of humanizing glycosylation pathways in various expression systems to produce human-like glycoproteins [187,188]. Glycosylation engineering of mammalian cell lines by stable transfection with genes encoding terminal human glycosyltransferases allows to obtain products with tailored glycosylation in high yields [189]. Umana et al. showed that the efficacy of antibodies could be improved by enhancing the potency of their natural immune effector functions [190]. Stable overexpres-

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sion of n-acetylglucosaminyltransferase III, an enzyme not naturally expressed in CHO and NSO cells, in recombinant antibody-producing cells generate IgG with high levels of bisected, non fucosylated oligosaccharides in the Fc region [190]. On the other hand, the CHO cell line has been engineered to express the missing enzyme α,2–6 sialylytransferase [191], and feeding with galactose has proven to improve galactosylation of produced antibodies. Insect cells, like other eukaryotic cells, modify many of their proteins by N-glycosylation. However, the endogenous insect cell N-glycan processing machinery generally does not produce complex, terminally sialylated N-glycans such as those found in mammalian systems [188]. Similarly, glycoengineering has been used to improve the baculovirus-insect cell system [192].

In plant, N-linked glycans may be processed to include potentially antigenic and/or allergenic β(1,2)-xylose residues attached to the α-linked mannose of the glycan core and α(1,3)-fucose residues linked to the proximal Glc-NAc [193]. However, recent studies suggest that these glycans have limited immunogenicity. Even if plant glycan patterns may not represent a problem in terms of human health, they may affect conformational epitopes, or clearance of plant derived antibodies. In attempt to "humanize" glycans on antibodies produced in plant, the human β-1,4-galactosyltransferase was stably expressed in tobacco plants [194].

Most efforts to humanize yeast glycosylation pathways [195] have focused on the deletion of specific yeast genes involved in hypermannosylation (α-1,6-mannosyltransferase Och1p), and the introduction of genes catalysing the synthesis, transport and addition of human sugars (α-1,2 mannosidase, galactosyltransferases) [196]. This approach has been successfully used in *Pichia pastoris* [197,198].

6. Recombinant antibodies: to the infinity and beyond...

Recent statistical data, concerning recombinant products entering the market, indicates that at least one out of three molecules is an antibody or an antibody-derived molecule. It is reasonable to think that the current tendency is promised to increase in the coming years. This can be explained by a favourable conjunction between a sustained demand from the market and the relative maturity of the antibody engineering techniques. In such a context one should not underestimate the tremendously complex patent situation governing the recombinant antibody world. Nevertheless, for scientists as well as for the pharmaceutical industry, the antibody is an inexhaustible source of inspiration. Multi-faceted protein, the antibody has been able to undergo successive metamorphosis making it always fitted with the needs of the medical and industrial worlds.

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Abbreviations used in this paper:

AAR: anti-antibody response, Ab: antibody, ADCC: antibody-dependant cellular cytotoxicity, APC: antigen presenting cell APEX: anchored periplasmic expression, BsAb: bispecific antibody, CAD: covalent antibody display, CDR: complementary determining regions, cGMP: current good manufacturing practice, CHO: chinese hamster ovary, EBV: Epstein-Barr virus, Fab: antigen-binding fragment, Fc: crystallisable fragment, FDA: Food and Drug Administration, H: Heavy chain, HACA: human anti-chimeric Ab, HAHA: human anti-humanized Ab, HAMA: human anti-mouse Ab, HCAb: Heavy chain antibody, HEK: Human embryo kidney, L: Light chain, mAb: monoclonal antibody, NAR: new antigen receptor, NK: natural killer; PBL: peripheral blood lymphocyte, PEG: polyethylene glycol, PSMA: prostate specific membrane antigen, scFv: single-chain Fv fragment, SCID: severe combined immunodeficient, SIP: selectively infective phage, TNF: tumor necrosis factor, YAC: yeast artificial chromosome.

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I, Andrew John Webb of 51 Cheney Street, Eastcote, Middlesex, HA5 2TA, UK declare as follows:

1. Qualifications

1.1. I have practised as a solicitor in IP matters for over 35 years. My relevant experience includes advising on contracts relating to technical and commercial confidential information such as Non-Disclosure Agreements ("NDAs") and Know How Licences and representing parties in litigation relating to breach of confidentiality obligations. I joined J A Kemp (now J A Kemp LLP) in 1991 at which point I additionally qualified as a Chartered Patent Attorney and European Patent Attorney and have practised as such. I am familiar with the European Patent Convention and EPO Case Law. One of my roles at J A Kemp was to provide legal support to my colleagues on European Patent matters in the manner of a Legally Qualified Member assisting the EPO Technical Members. In that capacity I have provided support at all levels up to and including the Enlarged Board of Appeal. I retired from J A Kemp LLP at the end of 2019. I remain a solicitor entitled to practice in the UK and I provide consultancy on an ad hoc basis to J A Kemp LLP.

2. Summary

2.1 I have been asked to review certain aspects of the Examining Division decision of 28 January 2020 concerning EP16172335.8 of Alexion Pharmaceuticals, Inc.

2.2 I conclude:

- The supply of eculizumab for clinical trials was for use in those trials in accordance with the trial protocol and the recipients were not free to use the product provided for any other purpose. The recipients were not free in equity and law to use the product for conducting sequence analysis and there is no evidence that they did so in breach of their obligations. That is sufficient to overcome the Examining Divisions objections;

- Even if the recipients had analysed the product, any information the recipients discovered concerning the product accrued to Alexion and was subject to restrictions on use and disclosure;

- The restrictions on use and disclosure applying to new information relating to the correct amino acid sequence of eculizumab, information not in the public domain, were not proscribed by availability of the CAS disclosure of the incorrect sequence. The restrictions on use and disclosure were imposed by contract. The question of what the recipient believed to be confidential does not have any impact on this contractual obligation.

3. The Examining Division Position

3.1. Oral Proceedings Minutes:

> *In particular, the legal member emphasised that the confidentiality agreement clearly excluded any information from the confidentiality obligation that had already become public. Since the sequence had been published by CAS with the intention to lay the structure open any signatory to the agreement must have understood it as excluding the sequence from confidentiality.*

3.2. Decision

> 5.1.2 *The examining division is of the opinion that confidentiality agreements – irrespective of the applicable national law they are subject to – cannot relate to information that is (believed to be) in the public domain. The scientists working with eculizumab were under the impression that the structure of eculizumab had been submitted to CAS. Hence, they were not bound to confidentiality with regard to the structure of eculizmab* (sic) *and could have performed a sequencing of the antibody with the general knowledge available in the art without technical or legal restraints…The examining division thus concludes that the structure of eculizumab was at least available to the scientists working with eculizumab without these scientists being bound to confidentiality in this respect… This line of argument…is also supported by the wording of the confidentiality agreements…In all instances (alleged) signatories to the agreements are bound to confidentiality only with regard to unpublished information or information which has not entered the public domain.*

4. Observations on the Examining Division Position

4.1. As discussed below:

- The evidence is that the recipients were provided with the product for a specific purpose, they were not free to use the product for other purposes and thus were not free to sequence the product;

- I am not aware of any rule of law which suggests that placing a piece of information in the public domain should interfere with the right to protect new information, such as new information correcting or adding to the previously disclosed piece of information.

5. The evidence on permitted use, ownership of information and confidentiality

5.1. For the purposes of preparing this declaration I have reviewed the EPO file and considered the evidence presented, bearing in mind the EPO free evaluation of evidence principals.

5.2. The principle evidence is provided in the Bombara declaration (D36) and its Exhibits, supported by the declarations of Leonard Bell (D31) and Laural Boone (D13),

Redacted study protocol for trial C04-001 (D22), and Redacted protocol for trial C02-002 (D23).

5.3. The Bombara declaration (D36) outlines the approach taken by Alexion, provides details for one particular study and confirms similar documents would have been executed for all the other eculizumab-related trials in the late 1990s and early 2000s.

Master Clinical Study Agreement

5.4. This is the top level agreement. An example is the MCSA at Exhibit C to Bombara declaration. It includes the following provisions:

5.5. **Clause 2 Data and Inventions** - …. All data, inventions, and discoveries …which… relates to a Study Drug shall belong solely and exclusively to Alexion.

5.6. **Clause 7 Confidentiality and Publication** - Institution agrees and shall cause each Principal Investigator and its employees and agents, not to disclose to any third party and not to use for any purpose other than conducting a Study…..data, inventions and discoveries which …..relate to a Study Drug. The preceding obligations shall not apply to information, data, inventions, or discoveries which have entered the public domain…

5.7. The agreement is expressed to be subject to English law.

Study Specifications

5.8. These are appended as Exhibit II to an MCSA. This includes, at Clause 8, agreement by the Principal Investigator to be bound by MCSA Clauses 2 and 7.

Study Protocol

5.9. Clauses 6.2 to 6.6 specify the supply, use and accountability requirements for eculizumab. Clauses 8 to 10 specify in detail the test treatment procedures. Eculizumab is provided for a specific limited purpose, to carry out the tests in accordance with the test protocol. This does not permit the recipient to use the supplied product for any other purpose. The recipient is not free to use the product supplied for any other purpose and must account for all product provided.

6. The State of the Art

6.1. Art. 54(2) EPC the state of the art shall be held to comprise everything made available to the public by means of a written or oral description, by use, or in any other way, before the date of filing of the European patent application.

6.2. To determine whether an invention has been made available to the public by prior use it is necessary to consider three questions: when, what and how.

7. Prior use questions

7.1. **When**: In the present case there is no dispute that before the filing date of the application eculizumab was provided to a number of institutions for the purpose of conducting specific clinical trials (D31 declaration of Leonard Bell, paragraph 2; D13 declaration of Laural Boone, paragraphs 3 to 6;

7.2. **What**: the research institutes were provided with eculizumab having the relevant light and heavy chain sequences (D13 declaration of Laural Boone, paragraph 6). Eculizumab was provided (C02/001 Protocol para 6.2) in the form of eculizumab (h5G1.1-mAb) 10 mg/ml packaged as a solution for injection in 30 ml vials (containing 27.5ml of solution) packaged in bulk containers and shipped to the study site.

7.3. **How**: the supply was not a commercial supply permitting the recipient to make use of the eculizumab for commercial purposes such as to entitle the recipient to make free use of the same. The supply was a supply without charge to be used for specified purposes, administration to patients by IV infusion in accordance with an agreed clinical protocol (Study Protocol paras 8 to 10), for which the institute received payment. All Study Drug provided was subject to Drug Accountability provisions (Study Protocol para 6.6).

7.4. Viewed as a whole the agreements reflect the position that Study Drug and accompanying information were provided for the sole purpose of conducting the trials in accordance with the Study Protocol. Both information provided to the investigators and information derived by the investigators was protected by confidentiality provisions.

7.5. In a situation where a product which is not commercially available is provided for a specific use it is not necessary to specify contractually what it cannot be used for. It can be used only for the use for which it has been provided. In order to establish that the recipient was free to analyse the product the Examining Division should have considered whether the evidence was such as to establish that the recipients were free under the contracts to analyse the product. There is no discussion on this point recorded in the minutes of the oral proceedings. The evidence on this point is not addressed in the decision.

7.6. There is no evidence that any of the recipients made any use of the product other than the use expressly provided for in the protocol, and in particular no evidence that any of the recipients analysed the product.

7.7. Since use of product for analysis of its amino acid structure would be contrary to the terms on which it was provided and the recipients did not act contrary to those terms the correct amino acid sequence was not made available to the recipients.

7.8. Further, even if the evidence had established that the recipients were able to use Study Drug to carry out amino acid sequencing, the correct amino acid sequence

comprised a discovery not already in the public domain. In accordance with MCSA Clauses 2 and 7, such discovery relating to the Study Drug belongs solely and exclusively to Alexion and may not be disclosed. Whether any discovered information is in the public domain is a question of fact. The discovery, which might be expressed in the broad terms that the Study Drug did not have the amino acid sequence recorded with CAS as the amino acid sequence for eculizumab, was not information in the public domain.

8. Law of Confidence

8.1. There are three circumstances which give rise to an obligation of confidence:

- An obligation imposed by contract. Such an obligation may be enforced by an action for breach of contract. The scope of protection is defined in the contract. In the UK such an obligation may also be enforced by an action for breach of confidence under common law.
- An obligation implied because of the circumstances of disclosure – this is determined on the basis of whether a reasonable man standing in the shoes of the recipient of the information would have realised that the information was being given to him in confidence. Protection can extend to information which has the necessary character of confidentiality. In the UK such an obligation may be enforced by an action for breach of confidence under common law.
- An obligation implied because of the special relationship between the parties concerned – for example, employer/employee relationships.

8.2. In the present case there are obligations imposed by contract. Those obligations are enforceable by actions for breach of contract or for breach of confidence.

8.3. The law recognises that there are public policy considerations, such as restraint of trade, restraint of competition, which can make overbroad contractual confidentiality agreements unenforceable. The contract in the present case recognises this and sets limits on its scope. The limits on the scope for information derived by the investigators relating to the product are set out in MCSA Clause 7.

8.4. *The preceding obligations shall not apply to information, data, inventions, or discoveries which have entered the public domain through no fault of institution, or where Alexion agrees in writing to its use or disclosure or which was known by institution prior to the date of disclosure by Alexion hereunder.*

8.5. These are standard limitations to remove from the confidentiality obligations information which if sought to be protected might make the contract unenforceable. They would be recognised as providing basis for ensuring enforceability of the confidentiality obligations in respect of information of the type described, including "*data, information and discoveries which are generated as a result of any Study or that otherwise relate to a Study Drug*".

8.6. The question of whether information is in the public domain is a question of fact, to be determined by evidence. The evidence supports the position that any sequence data adding to or contradicting the CAS sequence data that might have been derived by the investigators from analysis of the Study Drug was not in the public domain. It would therefore fall outside the limitations on protection set out in the contract. It would have been protected by the confidentiality obligations.

8.7. The Examining Division decision is based on a statement of law to the effect that information which the recipient wrongly believes to be in the public domain cannot be protected. The Examining Division has not provided any support for this statement of law. It is not a position which as a practitioner I recognise.

8.8. The issue of what the recipient believed to be confidential is potentially of relevance to an implied obligation of confidence, but the applicant is not relying on an implied obligation. Even if the applicant were relying on an implied obligation I would expect a court to find that the reasonable man test would be met to imply an obligation of confidence for information that a Study Drug not yet available on the market has a different amino acid sequence to that expected from information in the public domain.

9. Conclusions

9.1. The evidence does not establish on the balance of probabilities that the investigators were able to analyse the amino acid sequence of eculizumab "*free of legal constraints*". There is no evidence that they did so in breach of their contractual obligations. The corrections to the sequence were not made available to the investigators by the supply to them of Study Drug.

9.2. Even if the sequence corrections had become available to the investigators as a result of analysis of the Study Drug, the evidence does not establish on the balance of probabilities that they would have been free to make use of or publish the information. The sequence corrections would be a discovery relating to the Study Drug falling within Clauses 2 and 7 of the MCSA and protected as the confidential information of Alexion.

Signed:



ANDREW JOHN WEBB
J A KEMP LLP

Date: 21 May 2020

DECLARATION BY DR ANITA HILL

I, Dr Anita Hill, of Leeds, U.K., declare as follows:

1. I am currently a Vice President, Clinical Development, Global Consultant for Hematology at Alexion Pharmaceuticals, Inc. Prior to joining Alexion, I was a Consultant Hematologist for Leeds Teaching Hospitals NHS Trust, UK and Honorary Clinical Associate Professor for the University of Leeds from April 2010. Prior to that, I worked as a Consultant Hematologist for Bradford Teaching Hospitals NHS Foundation Trust, where I was the lead for haematology clinical trials and research. I received my medical degree with distinctions and honours from the University of Sheffield, UK in 1996. I did my post-graduate training at Sheffield Teaching Hospitals NHS Trust, St. George's Hospital, London and the Royal Marsden Hospital, London until 1999. I also earned a Ph.D. from the University of Leeds in 2007. I am active in many clinical trials involving therapies for PNH, aplastic anemia and autoimmune haemolytic anaemias and am the chief investigator for many international trials for PNH. My CV is set out in Annex A.

2. During my employment at Leeds Teaching Hospital, I was a member of the team that conducted trials to study the drug eculizumab for the treatment of paroxysmal nocturnal haemoglobinuria (PNH). Two of the studies – the first a small pilot study and the second a larger, Phase III study named TRUMPH – were designated study numbers C02-001 and C04-001, respectively. My role in both studies was a research clinical fellow. I worked closely with Dr Peter Hillmen, who was the principal investigator in each study.

3. Before beginning the trials, I was provided with a copy of each of the protocols for the trials (a copy of which is in these proceedings as D31, Exhibit E and D22). These protocols provide instructions as to how the trials must be carried out, and how the study drug (eculizumab) must be treated during the trials.

4. It was my understanding when carrying out the studies that the study drug was provided solely for the purpose of carrying out the testing set out in the protocols. It was also my understanding that the study drug should not be used for any other purpose, for example, the drug should not be used for further research or analysis.

5. I have been involved in a number of clinical trials during my work at Leeds Teaching Hospital and throughout my career. In each case, it would be routine practice to put in place a clinical trial protocol in advance of starting any trial. It is also routine practice that such study protocols provide a complete set of instructions for carrying out the trial, and that clinicians should not deviate from those instructions. An obligation not to use study drug for purposes other than the specific tests described in the study protocol was therefore normal practice.

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DECLARED at Leeds
United Kingdom

This 5th Day of June 2020

DocuSigned by:
Anita Hill
Anita Hill, MBChB (Hons), Ph.D.
Signer Name: Anita Hill
Signing Reason: I approve this document
Signing Time: 05-Jun-2020 | 07:53:31 EDT
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THIRD DECLARATION BY DR BRUCE ANDRIEN

I, Bruce Andrien, Senior Director, Protein Characterization, of Alexion Pharmaceuticals, Inc., of 100 College Street, New Haven, CT 06510, United States of America, make the following factual declaration concerning the structure of therapeutic antibodies and how they are made:

1) I am Senior Director, Protein Characterization, of Alexion Pharmaceuticals, Inc. ("Alexion"). A copy of my full curriculum vitae was attached to my declaration of 23 May 2017. I also submitted a second declaration in this matter on 2 August 2018.

2) Through my work at Alexion over the past 18 years, I have become familiar with the structure of therapeutic antibodies and how such antibodies are made.

3) I understand that EP 16195355.9 is a European patent application whose parent international application was filed on March 16, 2007. I have read the Examining Division's decision dated 11 February 2020 refusing this application.

4) I understand the pending claim 1 of EP 16195355.9 reads as follows:

> "*An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4.*"

5) I understand that SEQ ID NO: 4 in EP 16195355.9 reads as follows:

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGAS
ENIYGALNWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISS
LQPEDFATYYCQNVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGT
ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

6) In my declaration of 23 May 2017, I explained that 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 that 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.

7) In my declaration of 15 August 2018, I explained that one skilled in the art would have known in 2007 that the leader peptide of SEQ ID NO: 4 would not be present in the mature antibody.

8) I understand that in point (a) of Section 1.2 of the decision, the Examining Division states:

> "*Nowhere in the application is it indicated that the sequence for which protection is sought has to be in its mature form, or that a leader peptide has to be cleaved off from the immature form of the light chain represented by originally filed SEQ ID NO: 3 or SEQ ID NO: 4*."

9) Although such a statement is missing from the application, this does not affect my opinion set out in my declaration of 15 August 2018 that that one skilled in the art would have known in 2007 that the leader peptide of SEQ ID NO: 4 would not be present in the mature antibody, which is based on basic general knowledge of antibody structure and production.

10) I understand that in point (b) of Section 1.2 of the decision, the Examining Division states:

> "*Moreover, even if the skilled person could have though that SEQ ID NO:3 and SEQ ID NO: 4 included a leading peptide (which is not conceded), it wouldn't have been possible to identify the exact length of the leading peptide without a certain level of doubt*."

11) I disagree with this statement. A skilled person looking at SEQ ID NOs: 3 and 4 would immediately know with certainty where the leader peptide ends and the mature polypeptide begins. As explained in my declaration of 23 May 2017, citing to "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, published in

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1991 by the U.S. Department of Health and Human Services and compiled by Kabat *et al.*, given the presence of an N-terminal methionine followed by a stretch of mainly hydrophobic amino acids, and the typical ending amino acids for a light chain leader peptide (RC) adjacent to the typical starting amino acids of the mature light chain (DIQM), one skilled in the art looking at SEQ ID NOs: 3 and 4 would have no doubt that these sequences begin with a leader peptide, and that the leader peptide ends at residue 22 and the mature polypeptide beings at residue 23.

12) Explaining this another way, as can be seen from Exhibit D of D14 "SIGNAL PEPTIDE OF HUMAN KAPPA LIGHT CHAINS" from "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, in every antibody light chain precursor polypeptide which contains the sequence Gly-Ala-Arg-Cys (GARC) at positions 19-22, these are the last four amino acids of the signal peptide (amino acids -4, -3, -2 and -1). Similarly, as can be seen from Exhibit E of D14 "HUMAN KAPPA LIGHT CHAINS SUBGROUP I" from "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, in all cases where the light chain precursor polypeptide contains the sequence Asp-Ile-Gln-Met (DIQM) at residues 23-26, these are the first four amino acids of the mature polypeptide (amino acids 1-4).

13) The amino acids at positions 19-22 of SEQ ID NO: 3 and SEQ ID NO: 4 are Gly-Ala-Arg-Cys (GARC), and the amino acids at positions 23-26 of SEQ ID NO:3 and SEQ ID NO: 4 are Asp-Ile-Gln-Met (DIQM). Thus, there would have been no doubt in the mind of one skilled in the art that SEQ ID NO: 3 and SEQ ID NO: 4 contain a signal peptide at positions 1-22 and that the amino acid sequence of the mature antibody is at residues 23-214 of SEQ ID NO: 4 or residues 23-131 of SEQ ID NO: 3.

14) I understand that in point (c) of Section 1.1.2 of the decision, the Examining Division states the following:

> "*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. This is particularly true in the present case since the application relates to the field of engineered antibodies*

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and not of naturally occurring human antibodies (such as those mentioned in the Kabat document mentioned in D14)."

15) One skilled in the art would recognize that engineered full-length monoclonal antibodies with a pre-defined sequence, such as the antibody described in claim 1, are made by recombinant expression of polypeptides having the specific heavy and light chain amino acid sequences in transfected cells. In 2007, as is still the case today, full-length antibodies are always produced in cells *in vitro* or in transgenic organisms (see Leffly & Sodoyer (2005), page 45, section 5, introduction), and I am not aware of any non-cell based methods for producing full-length antibodies. Production of antibodies in cells always results in the cleavage of the light chain signal peptide as explained in my declaration of 15 August 2018.

16) One skilled in the art would understand that the other methods of antibody production mentioned on pages 18-19 of EP 16195355.9 (the hybridoma method and isolation from phage antibody libraries) are used to generate and isolate antibodies that can *later* be sequenced. The skilled person would know that these methods cannot be used to generate a full-length engineered monoclonal antibody having a specified amino acid sequence in the first instance. Further, hybridomas are cells (see Laffly & Sodoyer, page 34, section 2, introduction), and the signal peptide is therefore cleaved during monoclonal antibody production using the hybridoma method. Also, phage antibody libraries contain only antibody fragments and are used for screening, rather than production (see Laffly & Sodoyer, pages 37-39, paragraphs 3.2.3 and 3.2.3.1).

17) Thus, one skilled in the art would not consider EP 16195355.9 to be suggesting that the antibody can be produced other than by expression in transfected cells. As I stated earlier, expression in transfected cells will always result in the cleavage of the signal peptide in SEQ ID NO: 4, and so one skilled in the art would never consider that protection was sought in EP 16195355.9 for an antibody having a light chain consisting of the whole of SEQ ID NO: 4.

18) I also understand in point (d) of paragraph 1.1.2 of the decision, the Examining Division states:

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"*It cannot be excluded with certainty that after production of the mature antibody in the transfected cells (which is only one possible way of producing the claimed antibody – see page 19 of the application), a sequence of amino acids is attached to the N-terminal amino acid.*"

19) Reading the specification of EP 16195355.9, I do not see anything that suggests adding a signal peptide back to the N-terminus of the mature antibody. Further, I am not aware of any instances where a signal peptide has been added to the N-terminus of a mature antibody after the antibody has been produced. Therefore, it is my view that the one skilled in the art would have had no reason to even consider adding a signal peptide back to the N-terminus of the mature antibody.

20) Moreover, one skilled in the art would be aware of the well-recognized problem of immunogenicity that is often associated with therapeutic antibodies. In order to try and minimize the immunogenicity of an engineered therapeutic antibody the skilled person would understand that the antibody should be designed to be as similar as possible to antibodies that are naturally circulating in the human body (see Laffly & Sodoyer, section 2 at pages 34-36). Since naturally-occurring antibodies that are circulating in a human body do not possess a signal peptide, for this additional reason one skilled in the art would not consider that the applicant intended to define a therapeutic antibody to which a signal peptide has been re-attached to the N-terminus of the mature therapeutic antibody.

DECLARED at New Haven, CT
United States of America

05-Jun-2020 | 16:50:58 EDT

This _____Day of May
2020

DocuSigned by:
Bruce Andrien
Signer Name: Bruce Andrien
Signing Reason: I approve this document
Signing Time: 05-Jun-2020 | 16:50:34 EDT
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Bruce A. Andrien, Jr., Ph.D.

AUXILIARY REQUEST 11

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 10

CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 9

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected CHO or NS0 cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 8

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. An antibody for use according to claim 1, wherein the transfected cells are CHO or NS0 cells.

3. A pharmaceutical composition comprising an antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

4. A pharmaceutical composition for use according to claim 3, wherein the transfected cells are CHO or NS0 cells.

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AUXILIARY REQUEST 7

CLAIMS

1. An antibody that binds C5, wherein the antibody is eculizumab and comprises a heavy chain consisting of SEQ ID NO: 2, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5, wherein the antibody is eculizumab and comprises a heavy chain consisting of SEQ ID NO: 2, for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 6

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

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AUXILIARY REQUEST 5

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST 4

CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST 3

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected CHO or NS0 cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST 2

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

2. An antibody according to claim 1, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

3. A pharmaceutical composition comprising the antibody of claim 1 or claim 2.

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AUXILIARY REQUEST 1

CLAIMS

1. An antibody that binds C5, wherein the antibody is eculizumab and comprises a heavy chain consisting of SEQ ID NO: 2.

2. A pharmaceutical composition comprising the antibody of claim 1.

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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

5 July 2019

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

Thank you for the summons to attend oral proceedings dated 27 March 2019. The Applicant wishes to make the following submissions in advance of the hearing scheduled for 5 September 2019.

The claims currently on file form the basis of our main request.

Three auxiliary requests are attached and we ask that the Examining Division consider the auxiliary requests in the event that the main request is considered unallowable.

Tracked-changes versions of the main request showing the amendments being made to derive the first two auxiliary requests and of the first auxiliary request showing the amendments being made to derive the third auxiliary request are attached.

I attach a full list of the documents cited in these examination proceedings. A copy of each of D24 to D31 is attached.

The present claims

Claim 1 of the present application is directed to:

> *"An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4."*

Claim 2 is directed to a pharmaceutical composition comprising this antibody.

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The present application discloses that SEQ ID NO: 2 is the full (variable and constant) heavy chain amino acid sequence of eculizumab, and that SEQ ID NO: 4 is the full (variable and constant) light chain amino acid sequence of eculizumab. This is confirmed in paragraph 6 of the Boone declaration (D13). SEQ ID NO: 2 is the mature sequence and does not include the N-terminal signal peptide. The heavy chain constant region of SEQ ID NO: 2 is a unique, engineered heavy chain constant region. SEQ ID NO: 4 is an immature sequence that includes the 22 amino acid N-terminal signal peptide, which is cleaved off during antibody production in a cell. The light chain constant region of SEQ ID NO: 4 is a kappa constant region.

The claims are therefore directed to the specific antibody, eculizumab, which obtained marketing approval under the trade name SOLIRIS® after the filing date of the present application (see D15/D16).

The structure of eculizumab was not disclosed in the prior art

The full heavy chain and light chain amino acid sequences of SOLIRIS® (eculizumab) were first disclosed in the present application. Prior to the filing date of the present application, it was not known that "eculizumab" was an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

D1-D5 do not disclose the structure of the claimed antibody

None of D1-D5 includes any structural information for "eculizumab".

D1 refers to "eculizumab" as being the tradename of the antibody "*h5G1.1-mAb*". D1 does not include any sequence information, but states that:

> "*Methods for the preparation of h5G1.1-mAb, h5Gl.l-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.*" (D1, page 5, left column last two lines to right column line 8).

U.S. Pat. No. 6,355,245 is D11, and Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401,1996 is D7 in the present proceedings.

D2 describes "eculizumab" as "*a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). No structural information is provided in D2, and it does not reference any other documents.

D3 describes "eculizumab" as "*a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components*[14,15]" (D3, left column page 553, third paragraph, first three lines). Reference 14 in D3 is a general publication relating to humanization of antibodies (Riechmann et al. (1988) "Reshaping human antibodies for therapy" Nature 332: 323-7), and reference 15 is D7 in the present proceedings. No structural information is provided in D3 itself.

D4 describes "eculizumab" as "*a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12)*" (D4, page 673, right column, third paragraph, first three lines). Reference 12 in D4 is D7 in the present proceedings. D4 also includes the CAS STN database number "CAS: 219685-50-4" on page 673. However, as discussed below, this database did not include the correct sequence for eculizumab at the filing date of the present application. No structural information is provided in D4 itself.

D5 defines "eculizumab" as "*a monoclonal antibody that blocks the complement system at C5*" (D5, page 849, left column, last two lines of answer to first question). No structural information for "eculizumab" is provided in D5.

Thus, D1 to D5 do not provide any sequences for "eculizumab", and only point to D7 and/or D11 as describing "eculizumab".

Neither D7 nor D11 discloses the full structure of the claimed antibody

D11 and D7 are referred to in the cited prior art documents as describing "eculizumab". However, neither D7 nor D11 discloses the amino acid sequences of SOLIRIS® (eculizumab) recited in the present claims.

D7 and D11 describe the cloning of the murine 5G1.1 antibody and the generation of humanised variable region sequences.

The only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

D7 discloses CDR grafted V_L and V_H sequences (h5G1.1VCH and h5G1.1VLC in Figure 1 of D7) and also V_L and V_H sequences comprising back mutations (h5G1.1VHC+F and h5G1.1VLC+F in Figure 1 of D7) and reports that an intact humanised antibody of the IgG4 isotype was constructed and produced (see page 1396, paragraph spanning columns of D7).

As discussed above, the present claims are directed to an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4, which are the sequences of the antibody in SOLIRIS® (eculizumab). This claimed antibody is **not** the IgG4 antibody described in D7. In particular, eculizumab does not have an IgG4 heavy chain constant region, but a unique, engineered, non-naturally occurring constant region sequence that has 14 different amino acid residues compared to the IgG4 sequence (4 in the CH1 domain, 6 in the hinge region and 4 in the CH2 domain - see paragraphs 4 and 5 of the declaration of Dr Leonard Bell (D12)).

Therefore, although D1 refers to D7 and D11, and D3 and D4 refer to D7, supposedly as describing "eculizumab", neither D7 nor D11 discloses the full heavy chain sequence shown in SEQ ID NO: 2 of the present application and the full light chain sequence shown in SEQ ID NO: 4 of the present application.

With regard to D11, the Examining Division has argued that:

> "*The description of the invention (see page 16, lines 19-24) refers to D11 and states that the document discloses the preferred whole antibody named eculizumab. That would mean that the eculizumab claimed in the patent and the one disclosed in D11 is the same antibody*" (page 7, first paragraph of the summons dated 23 March 2019).

Page 16, lines 24-25 of the present application states that: "*These antibodies disclosed in U.S. Patent 6,355,245 [D11] include a preferred whole antibody (now named eculizumab)*". However, it is necessary to look at D11 to see what it actually discloses, and despite the statement made in the present application, D11 does not in fact disclose the sequences of any whole antibody.

As discussed above, the only disclosure relating to full length antibodies in D11 is a general paragraph at column 45, lines 24-33 which describes in general terms how matched pairs of the variable regions (e.g., a VL and a VH region) of the various described antibody molecules, Fds and light chains may be combined with constant region domains to form full length antibodies of the invention.

The statement in the present application is therefore correctly interpreted as meaning that D11 discloses a genus of antibodies that includes the preferred antibody of the present invention (eculizumab), and not that this antibody is explicitly disclosed in D11. It is a matter of fact that D11 does not disclose an antibody with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 as presently claimed.

None of D1-D5, D7 and D11 provide an enabling disclosure of the claimed antibody

In view of the above, the skilled person would not be able to reproduce "eculizumab" based on the disclosures of any of D1-D5, D7 and D11. According to the established case law, a disclosure in a prior art document is novelty-destroying only if the teaching it contains is reproducible (see, for example, T 1437/07, Reason 25, and Guidelines for Examination G-IV-2).

Hence, none of D1-D5 can be considered to belong to the state of the art according to Article 54(2) EPC, at least in as far as they relate to "eculizumab", and consequently none of D1-D5 prejudices the patentability of the claimed invention.

None of the patents listed in D8 provide an enabling disclosure of the claimed antibody

D8 lists a number of Alexion patents and patent applications that refer to "eculizumab". None of the patents and patent applications listed in D8 provide any sequence information for "eculizumab". Insofar as these patents and patent

applications use the term "eculizumab", they refer back to D7 and/or D11 as shown in the attached table (D24).

As discussed above, neither D7 nor D11 disclose an anti-C5 antibody as presently claimed, with a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4. These full sequences of the antibody in SOLIRIS® (eculizumab) were not known in the art at the filing date of the present application. Therefore, the prior art disclosures in the patents and patent applications do not enable the skilled person to make the specific antibody that is now claimed.

In this regard, it should be noted that the patent and patent applications listed in D8 are not focused only on eculizumab. These patents and applications relate to complement inhibitors more broadly, including anti-C5 antibodies generally. As shown in D8, with one exception (EP 1720571, the patent granted from D10, which has been revoked (see below)), "eculizumab" is referred to only in dependent claims. Therefore, the fact that there is no enabling disclosure of the specific antibody "eculizumab" does not render all of these patents and patent applications insufficient across their whole scope. The insufficiency extends only to the specific dependent claims that mention "eculizumab."

Comments made by Alexion in D9 in the course of opposition proceedings regarding a different patent do not change what was in fact disclosed in the prior art

The Examining Division considers that D10 destroys the novelty of the present claims because the applicant stated in D9 that the structure of the antibody had been made available to the public on 14 February 1999. However, the statement made in 2013 by the applicant in the opposition proceedings against the patent derived from D10 does not change what was disclosed in D10 in 2005, and does not retroactively make the disclosure of D10, or any other prior art document, enabling in so far as it relates to "eculizumab".

The issue of sufficiency of disclosure of "eculizumab" was raised in the opposition proceedings against EP 1720571 (the patent derived from D10). The Opponent observed that:

> *"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)* [D11 in the present proceedings] *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)* [D7 in the present proceedings].
>
> ***However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.***

> *D9* [D11] *is a patent assigned to Alexion Pharmaceuticals, Inc. which relates to C-5 specific antibodies for the treatment of inflammatory diseases.*
>
> *In example 7, D9* [D11] *discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9* [D11] *lists a number of humanized, chimeric and non humanized ScFv, Fd and light chains. D9* [D11] *does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.*
>
> *D10* [D7] *is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10* [D7] *discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1380), 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 5G1.1 called h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col. 1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10* [D7].
>
> *In view of the above, none of the patent, D9* [D11] *or D10* [D7] *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."* (See section 7.1 of D21)

In defence of its patent, Alexion replied to this attack by asserting that the sequence for eculizumab was publicly available and stated (in D9) that:

> "A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4."*

The copy of CAS entry no. 219685-560-4 provided did not include any sequences, the identity of the sequences made available on 14 February 1999 was not investigated at that time, and this argument was not challenged.

In the preliminary opinion that accompanied the summons to oral proceedings dated 14 January 2014 (D25), the Opposition Division expressed the opinion that:

> "I*n the absence of evidence that* eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed" (paragraph 2.29 of D25).*

The position of the Opposition Division was not further challenged by Alexion or discussed at the oral proceedings (see Alexion letter of 17 March 2014 (D26), which does not include any arguments relating to sufficiency of disclosure), and EP1720571 was revoked for lack of inventive step (see the Decision of the Opposition Division dated 24 June 2014 (D27)).

The statement made in defence of EP1720571 should have no bearing on the current proceedings. It does not change the facts of what was disclosed prior to the filing date of the present application. Nor did Alexion gain any advantage from the statement made in connection with the opposition to that earlier patent.

In accordance with the opinion of the Opponent and Opposition Division in the opposition against EP1720571 that this patent does not provide a sufficient disclosure of "eculizumab", the application for EP1720571, D10, also does not provide an enabling disclosure of "eculizumab" and does not destroy the novelty of the present claims, which are directed to the unique, engineered antibody in SOLIRIS®.

The sequence of eculizumab was not available in the CAS database at the filing date

Heavy and light chain amino acid sequences were submitted to the CAS Registry in 1999 under the name "eculizumab", but, as evidenced by the Saxe Declaration, the sequences submitted to CAS at this time were **not** the sequences of the antibody (eculizumab) in SOLIRIS® to which the present claims are directed. The correct sequences for eculizumab were later submitted to replace the incorrect sequences in May 2009. These facts are not disputed.

However, one argument put forward by the Examining Division is that the availability of incorrect sequences for eculizumab on the CAS Registry database amounts to a public disclosure of the correct sequences for eculizumab because it was the intention of Alexion to make the sequence of eculizumab public at the time, such that Alexion would be obliged to provide the correct sequence of eculizumab to anybody who requested it. The Examining Division has referred to "*CAS rules*" as obliging Alexion to provide such information. However, the Examining Division has not provided evidence that any such rules existed, and Alexion is not aware of any. The fact therefore remains that the correct sequence of eculizumab was not made available in the CAS Registry prior to the filing date of the present application.

The statement by the applicant in D9 ("A *sequence for eculizumab was submitted to Chemical Abstracts Service (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry no. 219685-560-4.")* does not change the fact that the wrong sequence was included in the CAS database in February 1999 and the fact that this incorrect sequence was still the sequence listed for "eculizumab" in the CAS database at the filing date of the present application. The context of the statement in D9 is explained above. Rather than speculate as to the intent of Alexion in 1999, the proper inquiry should be what in fact was disclosed at the filing date of the present application and whether a person skilled in the art would have known or been able to determine the structure of eculizumab, as recited in the present claims.

The Boards of Appeal have considered the prior art effect of documents that contain mistakes. In particular, as stated in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, in T 412/91 the board held that:

> *"in principle, what constituted the disclosure of a prior art document was governed not merely by the words actually used in its disclosure, but also by*

> *what the publication revealed to the skilled person as a matter of technical reality. If a statement was plainly wrong, whether because of its inherent improbability or because other material showed that it was wrong, then - although published - it did not form part of the state of the art. Conversely, if the skilled person could not see the statement was wrong, then it did form part of the prior art.*"

Similarly, in the Case Law of the Boards of Appeal, 8th edition, I-C-4.9, it is noted that:

> "*In T 230/01 the board noted that a document normally forms part of the state of the art, even if its disclosure is deficient, unless it can unequivocally be proven that the disclosure of the document is not enabling, or that the literal disclosure of the document is manifestly erroneous and does not represent the intended technical reality. Such a non-enabling or erroneous disclosure should then not be considered part of the state of the art.*"

Applying this reasoning to the present case, either the skilled person would recognise that the sequences of eculizumab in the CAS database must be wrong, in which case the incorrect information is not part of the state of the art, or the sequences in the CAS database do not destroy the novelty of the present claims.

The Board in T 591/90 considered that it could be assumed that a skilled person would try to correct recognisable errors in a prior document if, using his general technical knowledge and consulting the reference literature, he could see at once that the information in question was not correct. (See Case Law of the Boards of Appeal, 8th edition, I-C-4.9.)

In the present case, the skilled person would be unable to correct the errors in the eculizumab sequences in the CAS database based on his common general knowledge and by consulting the literature.

The Saxe declaration (D17) explains that the heavy chain sequence submitted to CAS was much longer (650 amino acids) than the actual heavy chain sequence (448 amino acids). The annotated copy of the sequences originally submitted to CAS attached as D28 (obtained by searching for CAS Registry Number: 219685-50-4 on http://commonchemistry.org/ and annotated by hand) shows how extensive the errors are. In particular, the heavy chain sequence originally submitted to CAS contains three stretches of additional amino acids, has two single amino acid substitutions and is missing a stretch of 16 amino acids compared to the correct sequence. For comparison, an annotated copy of the corrected sequences for CAS Registry Number: 219685-50-4 is attached as D28a.

As discussed above, the skilled person would not have known that eculizumab has a uniquely-engineered heavy chain, and so the skilled person would not have known what the correct heavy chain constant region sequence of eculizumab should be.

Also, the light chain submitted to CAS in 1999 contains a single amino acid substitution compared to the correct sequence in the variable region (see D28 and D28a). The light chain variable region sequence shown in D7 is the same as the

incorrect sequence at this position, and so the skilled person would have had no way of knowing that there was an error in the light chain variable region sequence.

Accordingly, the errors in the eculizumab sequences are not errors that would have been recognisable, or at least correctable, by a person skilled in the art at the filing date.

It is worth noting how the EPO would have treated an earlier patent application in which the Applicant had erroneously included the incorrect sequence for eculizumab, such as that submitted to the CAS Registry in 1999. In such a situation, it would have been the intention of the Applicant to disclose the sequence of eculizumab to the public in the patent application. However, the Applicant would not have been allowed to correct the sequence merely because it was his intention to make the correct sequence public and because the correct sequence was necessary to claim eculizumab and meet the sufficiency requirements of Article 83 EPC. To the contrary, the sequence in such a patent application could later be corrected only if it was immediately obvious that the sequence contained an error and also what the correct sequence should be (Rule 139 EPC). In other words, the Applicant's intent would not have been taken into consideration. We refer in this regard to Board of Appeal decision T1111/02 in which there were errors in a sequence provided in the patent application that led to a finding of insufficiency because the incorrect sequences could not be corrected under Rule 88 EPC (now Rule 139 EPC). Accordingly, it is the disclosure itself - rather than the intent of the Applicant - that is controlling. The same should apply in the present case (regardless of what the intent of the Applicant was in 1999).

The fact remains that the CAS database did not contain SEQ ID NOs: 2 and 4 of the present application at the filing date, and the incorrect sequences for eculizumab submitted to CAS in 1999 do not destroy the novelty of the present claims.

Eculizumab itself was not publicly available at the priority date

Eculizumab used in the clinical trials was not available to the public

The clinical trials described in the prior art did not make the antibody eculizumab available to the public. The position taken by the Examining Division in this regard is inconsistent with established jurisprudence of the Boards of Appeal.

We are filing herewith a new Declaration of Michael Bombara (D31) who managed many of the clinical trials that Alexion conducted in the late 1990s and early 2000s, and whose responsibilities included ensuring that all of the necessary contacts between Alexion and the principle investigators and/or their institutions or study sites were in place (see paragraph 2 of D31). Bombara confirms that no member of the public should have been able to obtain Alexion's confidential information and/or study drug material (eculizumab) in the course of any of the clinical studies (see paragraph 17 of D31).

The Bombara declaration summarises Alexion's clinical trial practice prior to the present filing date and explains the multiple layers of confidentiality that were built into each clinical study to ensure that the principal investigators and study

institutions or sites clearly understood their obligation to maintain Alexion's information, including the study drug, in strict confidence (see paragraph 5 of D31).

Bombara refers to one specific example of the agreements in place for the pilot PNH study of 11 PNH patients referenced in D1 to D5 (the "C02-001" study) and provides evidence of the agreements in place for this study. The declaration confirms that the authors on each of D1-D3 and D5 were affiliated with Alexion or one of the two institutions bound by these agreements (see paragraph 15 of D31). Bombara also states that similar documents would have been executed for all of the other eculizumab-related studies conducted by Alexion in the late 1990s and early 2000s (see paragraph 16 of D31).

The Bombara declaration additionally explains that the investigators and institutions agreed to adhere to strict requirements set forth in the clinical trial protocol regarding the tracking, logging, storage and disposal of study drug material, and provides an example of a drug disposal form (see paragraphs 12 and 13 of D31).

As an initial point, the Examining Division is incorrect that "*none of the documents cited in respect of the present application relate to clinical trials*". All of D1 to D5 relate to clinical trials carried out under the control of Alexion:

- D2 and D3 describe a pilot study that was conducted in 11 PNH patients for 12 weeks. This study is the "C02-001" study as identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D5 describes an extension of the "C02-001" study during which the PNH patients were treated for 52 weeks. This extension study is the "E02-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D1 describes a further extension of the "C02-001" study during which the PNH patients were treated for two years. This extension study is the "X03-001" study identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31).
- D4, which discloses Alexion Pharmaceuticals, Inc. as its source (page 675, right column) is a review paper that describes a series of clinical trials that were conducted with eculizumab. All of these trials are studies identified in the table in the Boone declaration (D13) and Exhibit B of the Bombara declaration (D31):
    - The study in 117 patients with idiopathic membranous nephropathy is "C99-004".
    - The study in 40 rheumatoid arthritis patients is "C97-001".
    - The study in 209 rheumatoid arthritis patients is "C99-001".
    - The study in 368 rheumatoid arthritis patients is "C001-004".
    - The two month trial in 10 patients with dermatomyositis is "C99-006".
    - The phase II trial in membranous nephritis is "C99-004".
    - The trial in systemic lupus erythematosus is "C97-002".
    - The pilot and extension studies in 11 PNH patients are "C02-001" and "E02-001".

The Boone declaration (D13) explains that all of these trials were carried out under confidentiality agreements and that the eculizumab drug supplies used in the studies were tightly controlled. We have also provided direct evidence of the relevant

provisions in the "C02-002" and "C04-001" studies, by way of example. As is clear from paragraphs 7 to 9 of D13 and paragraphs 5 and 16 of D31, all of the studies listed in the table included substantively identical confidentiality provisions. It is also stated in paragraph 2 of the Bell declaration (D12) and paragraph 3 of the Bombara declaration (D31) that eculizumab was used in clinical trials before 15 March 2007 only under strict control and conditions of confidentiality.

The Examining Division is asking that we provide proof of a confidentiality agreement between Alexion and each participant/physician involved in the clinical studies. This level of proof is not, however necessary.

It is stated in Case Law of the Boards of Appeal III-G-5.1.1 that:

> *"In ex parte proceedings the applicant bears the burden of proof for the facts in his favour, e.g. the fact that a document cited by the examining division does not form part of the state of the art (**T 160/92**, OJ 1995, 35)"*,

However, this statement does not require that the Applicant provide absolute proof. The Case Law of the Boards of Appeal III-G-5.1.1 goes on, in the following paragraph to say:

> *"As far as issues relating to patentability requirements are concerned, the burden of proof in **examination proceedings** cannot lie initially with the applicant. If an examining division raises an objection, it must be appropriately substantiated.* In T **578/06** the *examining division had failed to provide such substantiation. Also, the passage in the "Case Law of the Boards of Appeal of the EPO" (see paragraph immediately above) could not support its view that in ex parte proceedings the applicant has the burden of proof for facts in his favour, as it appeared to have been taken out of its context. In this respect, the board clarified that all the procedural situations referred to in this passage were those where, in response to a substantiated objection from the examining division, the applicant was required to support its contention."*

Further, the same section of the Case Law of the Boards of Appeal (III-G-5.1.1) goes on to explain that:

> *"Where a lack of novelty is alleged, the burden of proof invariably lies with the party claiming that the information in question was made available to the public before the relevant date (see, for example **T 193/84**, **T 73/86**, **T 162/87**, **T 293/87**, **T 381/87**, OJ 1990, 213; **T 245/88** and **T 82/90**). According to the boards' established case law, each of the parties to the proceedings bears the burden of proof for the facts it alleges. If a party, whose arguments rest on these alleged facts, does not discharge its burden of proof, this is to the detriment of that party, who may not shift the onus of proof onto the other party (see **T 270/90**, OJ 1993, 725; **T 355/97**; **T 836/02**; **T 176/04**; **T 175/09**; **T 443/09**)."*

We have provided direct evidence of confidentiality agreements in some of the studies using eculizumab (D22, D23 and D31), and declaratory evidence that confidentiality provisions applied to all of the studies using eculizumab (D12, D13 and

D31). The Examining Division, on the other hand, has not substantiated its arguments that eculizumab was made available to the public as a result of the clinical trials carried out before the filing date, nor has the Examining Division provided any contrary evidence to that we have submitted.

The evidence we have provided does not include any confidentiality agreements with the patients taking part in the clinical trials. However, the patients were never in a position to inspect or distribute eculizumab. Eculizumab is administered by intravenous infusion in the clinic (see Declaration of Leonard Bell (D12), paragraph 2 and Declaration of Michael Bombara (D31), paragraph 3). Therefore, the intravenous administration of eculizumab is akin to the implantation of a prototype device (T152/03 and T906/01) and the eculizumab clinical trials can be distinguished from the trials in T7/07 where a large number of patients were given tablets to take home with them for use over a longer period of time (see T7/07 Reason 3.3). The eculizumab used in the trials carried out prior to the filing date of the present application remained under the strict control of health care practitioners who were bound by strict confidentiality obligations and was not made available or accessible to the public.

Further, it is established in T152/03 and T906/01 that the development and test phases of implanted products were necessarily surrounded by secrecy as long as such products had not been commercialised, and that there was a prima facie assumption that any person involved in a medical tests of such products was obliged to maintain confidentiality and that any evidence proving the contrary was important. In the present case, eculizumab had not been commercialised at the filing date (see D13, paragraph 14, D15 and D16). There should therefore be an assumption that those involved with the trials were obliged to maintain confidentiality. Furthermore, we have provided evidence that the trials were carried out under confidentiality agreements with Alexion and that eculizumab used in the clinical trials remained under the strict control of Alexion (see D12, D13 and D31).

As stated in the Case Law of the Boards of Appeal, 8th edition, III-G-4.3.1:

> *"The EPO departments decide on the issues that arise before them on the basis of the evidence adduced by the parties. Their decisions need not, and in most cases cannot, be based on absolute conviction, but instead are to be arrived at on the basis of the overall balance of probabilities, in other words on the footing that one set of facts is more likely to be true than the other. If the result of the boards' evaluation of the evidence is such as to persuade them one way or another, then the* ***balance of probabilities*** *standard is met. This standard applies particularly in opposition appeal proceedings where the boards of appeal are called upon to reach a conclusion on the basis of the overall balance of probabilities, as distinct from "beyond all reasonable doubt" or "absolute conviction" (on the latter concept, refer to the more recent case* ***T 2451/13****). Each of the parties must therefore seek to prove facts alleged by it to that degree of proof (see e.g.* ***T 182/89****, OJ 1991, 391;* ***T 270/90****, OJ 1993, 725;* ***T 859/90****,* ***T 109/91****,* ***T 409/91****, OJ 1994, 653;* ***T 1054/92****,* ***T 296/93****, OJ 1995, 627;* ***T 326/93****,* ***T 343/95****,* ***T 363/96****). The standard of balance of probabilities applies also in decisions issued in ex parte proceedings (****T 381/87****, OJ 1990, 213;* ***T 69/86****,* ***T 128/87****, OJ 1989, 406;* ***T 939/92****, OJ 1996, 309).*

> *When an issue of fact is being examined and decided by the EPO on the balance of probabilities,* ***the more serious the issue*** *the more convincing must the evidence be to support it. If a decision on such an issue may result in refusal or revocation of a European patent, for example, in a case concerning alleged prior publication or prior use, the available evidence in relation to that issue must be very critically and strictly examined. A European patent should not be refused or revoked unless the grounds for refusal or revocation (that is, the legal and factual reasons) are fully and properly proved (****T 750/94****, OJ 1998, 32;* ***T 329/02****). See also Guidelines G-IV, 1 "State of the art – General remarks and definition" – November 2015 version): "If the applicant shows sound reasons for doubting whether the document forms part of the 'state of the art' in relation to his application and any further investigation does not produce evidence sufficient to remove that doubt, the examiner should not pursue the matter further."* [emphasis added]

The evidence we have submitted shows that the clinical trials using eculizumab were carried out under confidentiality agreements and that Alexion imposed strict controls on the eculizumab used in the trials. The eculizumab used in the trials was not available to the public. There was no public prior use of eculizumab, and the documents that describe the clinical trials that refer to eculizumab only by name (D1-D5, D10 and the patents and applications listed in D8 that have an earlier filing date that the present application) do not provide an enabling disclosure of eculizumab.

Use of the term "eculizumab" is not evidence that it was publicly available

The Examining Division is incorrect that the fact that many patent and non-patent documents in the prior art related to the use of "eculizumab" is evidence that the compound is well-known and available to the public.

It is established Board of Appeal case law that simply mentioning a biological material by name in a document does not make it publicly available. As stated by the Board in T576/91, if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "available to the public" within the meaning of Article 54(2) EPC.

In this case, access to eculizumab was limited to the physicians involved in the trials who had signed confidentiality agreements with Alexion, agreed to store eculizumab in a secure, limited-access area and agreed to strict provisions regarding the tracking and disposal of any unused eculizumab. The mention of "eculizumab" by name in public reports of the trials and in patent applications is therefore not evidence that eculizumab was publicly available.

Thus, the absence of a statement in D1-D5 that eculizumab had not been "*available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals*" does not mean that the antibody was available to the public. The evidence shows that the working group using eculizumab was bound to a confidentiality agreement with Alexion Pharmaceuticals. Therefore,

in accordance with the case law (T576/91), it cannot be concluded that eculizumab had been made "available to the public" within the meaning of Article 54(2) EPC.

Articles 76(1) and 123(2) EPC

The amendment to refer to a light chain consisting of residues 23-214 of SEQ ID NO: 4, and to a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 is an obvious correction that meets with the requirements of Rule 139 EPC.

The Examining Division considers that:

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and the application as originally filed were actually those for which protection was sought."*

The Examining Division notes that neither the original application nor the parent application contain a disclosure of the fact that SEQ ID NOs: 3 and 4 did not have to include the leader peptide. Such an indication is not, however, necessary because it was part of the skilled person's common general knowledge that antibodies do not include leader peptides. Evidence in support of the skilled person's common general knowledge has already been filed as D14, D19 and D20.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> *"The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC."* (Head note 1; emphasis added)

The Examining Division talks in terms of protection being sought for SEQ ID NOs: 3 and 4. However, protection of these sequences is not what is sought. Rather, it is protection for an antibody defined in terms of its sequences that is sought by the Applicant. Therefore, it is not correction of SEQ ID NOs: 3 and 4 that is requested. Rather, correction is requested only of the definition of an antibody defined with reference to these sequences.

It would have been immediately apparent to the skilled person that the eculizumab light chain sequences shown in SEQ ID NOs: 3 and 4 of the original application and of the parent application include a 22 amino acid signal peptide at the N-terminus. The skilled person would know from his common general knowledge that the 22 amino acid signal peptide in SEQ ID NOs: 3 and 4 would be cleaved off during antibody production in the cell.

The skilled person would consequently immediately recognise that the recitation at page 5, lines 30-33 of the original application and of the parent application of an antibody that comprises a light chain that "*consists of SEQ ID NO: 4*" is an error, and

that the recitation at page 5, lines 25-28 of an antibody having a light chain variable region that "*consists of SEQ ID NO: 3*" is an error.

The skilled person, based on his common general knowledge, would also immediately know that it was the Applicant's intention to define the antibody as including only the mature light chain sequence, i.e. residues 23-214 of SEQ ID NO: 4 or residues 23-109 of SEQ ID NO: 3.

The correction to define the antibody only by reference to the mature light chain sequence is therefore an obvious correction in accordance with Rule 139 EPC, and the present application meets the requirements of Articles 76(1) and 123(2) EPC.

Sufficiency - Article 83 EPC

The insufficiency objection raised by the Examining Division arises because the Examining Division does not accept that the correction made to define the antibody in terms of the mature heavy and light chain sequences is an obvious correction of an obvious error. However, the Examining Division has misinterpreted the Applicant's arguments. The application does not include the wrong sequences. SEQ ID NOs: 3 and 4 are the correct light chain variable and full light chain sequences, respectively, but they contain the 22 amino acid signal peptide that is cleaved off during antibody production and so is not present in the mature antibody which comprises two heavy chains and two light chains. Accordingly, the sequences filed in the original application do identify the structure of eculizumab.

The question to ask when determining whether the disclosure of the application is sufficient is whether the skilled person could make the claimed antibody without undue burden based on the teaching in the patent.

Claim 1 defines the antibody by reference to its full mature heavy and light chain sequences. It is routine for the skilled person to make an antibody having known full mature heavy and light chain sequences. Therefore, there is no question that the skilled person would be able to make the antibody of claim 1 without undue burden.

Inventive Step - Article 56 EPC

The Examining Division has identified the difference between the "*available documents of the prior art describing eculizumab (D1-D5, D10 and D11)*" and the claimed antibody as being the sequence of the claimed compound. The Examining Division refers to the incorrect CAS database entry for eculizumab as was available at the filing date. It is argued that the contribution of the patent is correction of an error in a registered sequence listing, which is devoid of technical character. The Examining Division then asserts that the skilled person had the knowledge to "*carry out the sequence of eculizumab (see the teaching of D7)*". This is not the correct application of the problem and solution approach.

As discussed above, none of D1-D5, D7, D10 and D11 provide an enabling disclosure of "eculizumab". Therefore, the teachings of these documents in so far as they refer to "eculizumab" are not part of the state of the art under Article 54(2) EPC (T1437/05).

The present application is concerned with providing treatments for PNH. Therefore the closest prior art document is one that is also concerned with treating PNH.

D1 is concerned with treating PNH, and other haemolytic diseases, using inhibitors of complement components, such as complement-inhibiting antibodies. D1 describes a clinical trial using the specific anti-C5 antibody "eculizumab". D1 does not disclose the sequence of "eculizumab", but cites D7 and D11 as describing suitable anti-C5 antibodies including "eculizumab". As explained above, D11 does not disclose the full sequence of any antibody and the only full length antibody described in D7 is an IgG4 isotype antibody. Therefore, the teaching in D1 would have led the skilled person to an antibody having a naturally occurring IgG4 heavy chain constant region. Thus, whilst D1 does not provide an enabling disclosure of "eculizumab" for treating PNH, it does provide an enabling disclosure of the IgG4 antibody disclosed in D7 for treating PNH.

In contrast, the antibody of claim 1 has an engineered heavy chain constant region. The amino acid differences between the IgG4 heavy chain constant region and the engineered heavy chain constant region of the antibody of claim 1 are shown in the Bell declaration (D12) in paragraph 5. The technical effects of these differences are also set out in D12. Paragraph 6 of D12 explains that differences in the heavy chain constant region result in the antibody having reduced binding to Fcγ receptors such that immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway are minimised (see also the experimental report, D18). Paragraph 7 of D12 explains that differences in the heavy chain constant region result in the antibody having an increased plasma half-life (see also the experimental report attached as D29).

The objective technical problem in view of D1 may therefore be defined as the provision of an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH. The solution is solved by the presently claimed antibody. The solution provided would not have been obvious to the skilled person at the filing date.

D7 explains why the IgG4 isotype was chosen for the full length antibody, reporting that:

> "*The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao et al., 1993) and there is only one known allotype of IgG4 (Ghanem et al., 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10)*" (D7, page 1399, right column, second paragraph, lines 15-25)."

The skilled person would have had no motivation to use an antibody different than the IgG4 antibody described in D1/D7 in view of the report in D1 of the successful treatment of PNH, and the teaching in D7 discussed above that the human IgG4 isotype has the advantages that it does not activate human complement and has only

one known allotype, precluding an immune response against the constant region of the antibody.

In particular, the skilled person would not consider using a different, engineered constant region sequence in place of the IgG4 isotype antibody disclosed in D7 because he would be concerned about the immunogenicity of the therapeutic antibody, and D7 teaches that the IgG4 isotype is ideal in this regard. The safety of the IgG4 antibody of D7 would have been confirmed to a skilled person by the clinical studies reporting that "eculizumab" (identified as the antibody of D7) was safe and effective when administered to PNH patients (e.g. D1, D3 and D4). Also, at the filing date of the application, other IgG4 isotype antibodies had been commercialised as therapeutic antibody products (see D30, table 1). Significantly, at the filing date of the application, no antibody having the uniquely-engineered heavy chain constant region of SEQ ID NO: 2 was on the market or publicly known to be studied in human clinical trials.

Furthermore, the advantageous properties of reduced immunogenicity and increased half-life exhibited by the claimed antibody are not taught or suggested in any of the cited documents. Indeed, in paragraph 8 of D12, it is explained that the advantageous properties of the claimed antibody over the antibody of D7 (which is the antibody to which D1 relates) were surprising and unpredictable.

Accordingly, the solution to the problem of providing an antibody having improved properties compared to the IgG4 antibody of D7 for therapeutic use in treating PNH provided by the present claims would not have been obvious to the skilled person at the filing date, and the claims involve an inventive step.

First Auxiliary Request

In the first Auxiliary Request, the antibody is defined as "*comprising the heavy chain amino acid shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4*". Basis for this amendment may be found in the originally filed application at page 4, line 32 and page 44.

Second Auxiliary Request

The second Auxiliary Request is the same as the Main Request, except that claim 1 has been rewritten as a purpose-limited product claim reciting the treatment of PNH.

Third Auxiliary Request

The third Auxiliary Request contains the amendments made to the first and second Auxiliary Requests.

Conclusion

It is believed that this application is in allowable form and the Examining Division is requested to reconsider it favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. The Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be

allowed without the need for Oral Proceedings on 5 September 2019, on the basis of the Main Request or, failing that, on one of the Auxiliary Requests. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
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BY ONLINE FILING

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16 January 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

In response to the Communication pursuant to Article 94(3) EPC dated 17 November 2017, please replace the claims currently on file with the new claims attached herewith. Please also replace description page 5 with new description page 5 attached herewith.

To assist the Examiner, a tracked changes version of the claims and a tracked changes version of description page 5 showing the amendments being made are also attached.

Also attached are: (i) a copy of a letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007; (ii) a copy of a press release made by Alexion Pharmaceuticals on 16 March 2007; (iii) a Declaration of Stephen Saxe; and (iv) an Experimental Report.

Amendments

Claims 1 and 2 have been combined as new claim 1. An obvious error in referring to the whole of SEQ ID NO: 4 in the definition of the light chain of the antibody has been corrected.

A corresponding correction has been made at page 5 of the description.

Correction of Obvious Error

In the response of 10 July 2017 to the Search Opinion, correction of the sequence listing was requested. In the corrected sequence listing, the leader peptide was deleted from SEQ ID NO: 3 and SEQ ID NO: 4. The Examining Division has determined

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that this correction of the sequence listing is not allowable under Rule 139 EPC because it considers that there is no indication in the original application that SEQ ID NO: 3 and SEQ ID NO: 4 did not have to include the leader peptide.

Rather than correcting the sequence listing, we now request correction of the statements in the description that:

> "*In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3*"; and
>
> "*In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO: 2 and the light chain consists of SEQ ID NO: 4*".

It would be immediately evident to the skilled person that an antibody light chain variable region could not consist of SEQ ID NO: 3 and that the light chain of an antibody could not consist of SEQ ID NO: 4.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "*The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1)*

Reading the present application, the skilled person would immediately recognise, based on his common general knowledge, that each of SEQ ID NO: 3 and SEQ ID NO: 4 contain a leader sequence as explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien.

The skilled person would also know that leader sequences are not present in an antibody ready for binding an antigen because these sequences are cleaved off during antibody maturation and production in cells. Therefore, it would be immediately evident to the skilled person that the references at page 5 of the description to an antibody comprising a light chain variable region consisting of SEQ ID NO: 3 and an antibody comprising a light chain consisting of SEQ ID NO: 4 represent an error.

As previously explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien, using his common general knowledge, the skilled person would have no difficulty determining which of the amino acids shown in SEQ ID NO: 3 and SEQ ID NO: 4 correspond to a leader peptide.

Therefore, the corrections being made to page 5 of the description to refer to the start and end positions of the light chain variable region and the light chain of the antibody would immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC.

It follows that the reference to the start and end positions of the mature part of the light chain sequence in SEQ ID NO: 4 in claim 1 complies with the requirements of Article 123(2) EPC.

Novelty

Claim 1 is novel because there was no public prior use of eculizumab before the filing date of the present application and no prior art document contains an enabling disclosure of eculizumab.

Eculizumab was not available on the market at the filing date

Contrary to the assertion in the Examination Report, none of D1-D5 indicate that eculizumab was a product available on the market prior to the filing date of 16 March 2007.

D1 is a US patent application which describes therapy trials to evaluate the effects of an anti-C5 antibody on the rare disease PNH and symptoms associated therewith. At page 9, paragraph [0082] of D1 it is stated that "*the specific anti-C5 antibody used in the study was eculizumab*". However, D1 does not say where eculizumab was obtained from, and does not indicate that eculizumab was commercially marketed or otherwise available to the public.

D2 is an abstract describing a clinical trial showing that eculizumab appears to be the first effective therapy for haemolysis in PNH. D2 discloses that "*eculizumab is a human monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). Like D1, D2 neither indicates from where eculizumab was obtained, nor does it indicate that eculizumab was commercially marketed or otherwise available to the public.

D3 is a paper describing the effect of eculizumab on haemolysis and transfusion requirements in patients with PNH. D3 states that: "*eculizumab is a recombinant humanised monoclonal antibody that was designed to block the activation of terminal complement components*". Again, no indication as to where eculizumab was obtained from is given, and there is no indication that eculizumab was commercially marketed or otherwise available to the public.

D5 is a report of an interview with Anita Hill relating to the use of eculizumab for the treatment of PNH. D5 does not provide any information as to how eculizumab may be obtained, nor does it suggest that eculizumab was commercially marketed or otherwise available to the public.

D4 is a paper describing eculizumab. D4 states that "*eculizumab (5G1.1) is a recombinant, fully humanised monoclonal antibody that inhibits the activation of*

terminal complement components" (D4, Abstract, first sentence). D4 indicates that the source of eculizumab is Alexion Pharmaceuticals, Inc. (US) (D4 page 6-7, right column, third paragraph), but it does not indicate that eculizumab was commercially marketed or otherwise available to the public.

Eculizumab was, in fact, not commercially marketed or otherwise available to the public at the publication dates of any of these references. As explicitly stated in the Declaration of Laural Boone, a Senior Director and Patent Counsellor at Alexion Pharmaceuticals, Inc. filed with our response of 10 July 2017, eculizumab was first approved for commercial marketing and sale on 16 March 2007, which is the day after the filing date of the present application (see paragraph 14 of the Boone Declaration).

The Boone Declaration is further supported by the attached letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007, licensing Alexion Pharmaceuticals to manufacture the product eculizumab. The attached press release made by Alexion Pharmaceuticals on 16 March 2017 announcing that it had, that day, received marketing approval from the U.S. Food and Drug Administration (FDA) for eculizumab.

Therefore, it is a matter of fact that eculizumab was not a product available on the market at the filing date of the present application.

The structure of eculizumab was not publically known at the filing date

None of the cited documents disclose the full amino acid sequence of eculizumab.

Eculizumab is an antibody that does not correspond to any naturally occurring isotypes. Rather, eculizumab has a unique, non-naturally occurring, protein-engineered full heavy chain (including the CH1-hinge-CH2-CH3 regions). This unique heavy chain sequence is not disclosed in the prior art.

D7 provides sequence information for an anti-C5 designated antibody h5G1.1. Eculizumab is derived from h5G1.1. However, D7 only discloses the heavy and light chain variable region (VH and VL) sequences of the antibody (see Figure 1 of D7). Significantly, D7 does not disclose the full, uniquely-engineered heavy chain sequence of eculizumab. There is also an amino acid difference between the light chain variable region sequence shown in D7 and the light chain variable region sequence of eculizumab. A glutamine (Gln/Q) residue is present at position 60 of SEQ ID NO: 4 but an arginine residue (Arg/R) is present at this position in the light chain variable region sequence shown in D7 (position 38 of the mature sequence of "h5G1.1 VLC" in Figure 1 of D7).

Thus, D7 does not disclose an antibody comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.

D1, D2 and D5 describe eculizumab by name only; they do not disclose the structure of eculizumab.

D3 and D4 each refer to Thomas et al. (1996) Mol. Immunol. 33: 1389-401 (reference 15 in D3) which is cited as D7 in the Examination Report of 17 November 2017. As

discussed above, D7 does not disclose the full heavy and light chain sequences of eculizumab. Therefore, D3 and D4 do not enable the skilled person to make eculizumab.

The Examining Division notes that D6 indicates that eculizumab was entered in the CAS registry database on 14 February 1999. However, D6 is a copy of the CAS registry database on 6 March 2017. D6 cannot therefore be used as evidence that SEQ ID NO: 2 and SEQ ID NO: 4 were available to the public ten years earlier at the 15 March 2007 filing date.

The sequence information for eculizumab submitted to the CAS Registry on 14 February 1999 was incorrect. The correct sequence information for eculizumab was not entered on the registry database until May 2018, which is after the filing date of the present application.

The attached Declaration of Stephen Saxe, and its annexes, show that the sequences for CAS Registry Number 219685-50-4 as shown in D6 were not available in the CAS Registry database until May 2009. In particular, Annex C, the email sent from Lois Bishop at CAS Client Services to Stephen Saxe on 1 May 2009 confirms that changes were made to the sequence for CAS Registry Number 219685-50-4 that would appear on STN a couple of days after that email was sent. Annex B, an email and letter sent from Stephen Saxe to the CAS, shows that the CAS Registry Number 219685-50-4 heavy chain sequence in the database prior to May 2009 was 650 amino acids in length, whereas SEQ ID NO: 2 is a 448 amino acid sequence, and that a single error was corrected in the light chain sequence (see paragraph 3 of the letter and point 3 of the Saxe Declaration).

Thus, the heavy chain sequence in the CAS database at the filing date of the present application contained hundreds of amino acids that are not present in SEQ ID NO: 2 and there is one amino acid difference between the light chain sequence in the CAS database at the filing date of the present application and residues 23 to 214 of SEQ ID NO: 4. Therefore, D6 is not evidence that an antibody comprising the heavy chain sequence shown in SEQ ID NO: 2 and the light chain sequence shown in residues 23 to 214 of SEQ ID NO: 4 was available to the public at the filing date of the present application.

In addition, as set out in the Declaration of Laural Boone, the heavy and light chain sequences of eculizumab (SEQ ID NOs: 2 and residues 23 to 214 of SEQ ID NO: 4 in the present application) were not made available to doctors or patients participating in any of the trials using eculizumab prior to at least 16 March 2007 (see paragraphs 6 and 7 of the Boone Declaration).

Thus, the full amino acid sequences of the heavy and light chain sequences of eculizumab were not disclosed in any of the cited documents.

The skilled person would not have been able to analyse the structure of eculizumab and reproduce it

In G1/92, the Enlarged Board of Appeal concluded that the chemical composition of a product is state of the art when the product as such is available to the public and can be analysed and reproduced by the skilled person. However, none of the studies

using eculizumab as described in D1-D5 made eculizumab available to the public such that the skilled person would have been able to analyse the structure of eculizumab and reproduce it.

As set out in the Declaration of Laural Boone, all physicians and participants in the eculizumab trials were required to maintain Alexion's confidential and/or proprietary information in strict confidence (see paragraphs 6 and 7 of the Boone Declaration). The clinical trials required strict controls to be placed over the use of eculizumab as explained in paragraphs 10 and 11 of the Boone Declaration. In particular, all eculizumab not administered to patients in the trials had to be accounted for (paragraph 10 of Boone Declaration).

Therefore, the skilled person would not have been able to obtain and analyse eculizumab to determine its structure, and would not have been able to reproduce eculizumab at the filing date of the present application.

Third Party Observations

The third party observations filed on 12 December 2018 are merely circumstantial and do not provide any evidence that eculizumab was publically available at the filing date of the present invention.

Summary

Although reports of trials describing eculizumab were available at the filing date of the present application, eculizumab was not made publically available until after the filing date. Eculizumab was not on the market at the filing date; the full structure of eculizumab was not published; and the skilled person would not have been able to analyse eculizumab and reproduce it.

Accordingly, there was no public prior use, public availability or enabling disclosure of eculizumab at the filing date. Claim 1 is therefore novel.

Inventive Step

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive. In particular, SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any information about the structure of eculizumab. Accordingly, it would not have been obvious to the skilled person from any of these documents to protein engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2. Accordingly, claim 1 involves an inventive step.

Sufficiency

The application provides a sufficient description of the claimed invention. SEQ ID NO: 2 is the complete heavy chain amino acid sequence of eculizumab, and residues 23 to 214 of SEQ ID NO: 4 represent the complete light chain amino acid sequence of eculizumab. Therefore, the complete structure of eculizumab is disclosed in the application.

In contrast, as discussed above, the full heavy and light chain sequences of eculizumab were not disclosed before the filing date of the present application. Thus, the skilled person reading any of D1 to D5 did not know the structure of the antibody that was actually tested and corresponded to the name “eculizumab.” However, in view of the inclusion of the full heavy chain sequence of SEQ ID NO: 2 and the full light chain sequence in SEQ ID NO: 4 (residues 23 to 214), the present application provides the first enabling disclosure of the antibody eculizumab.

Accordingly, the requirements of Article 83 EPC are met by the present application.

Concluding Remarks

It is believed that this application is now in an allowable form and the Examiner is asked to reconsider it favourably. If, however, there are any matters which remain outstanding, the Examiner is requested to issue a further Examination Report or telephone the undersigned. Merely as a precaution, in the event that the Examiner is minded to reject the application without first issuing a further Examination Report, oral proceedings are requested.

In addition, if the Examiner is minded to issue a Rule 71(3) Communication but wishes to propose amendments to the claims or description, it is requested that the Examiner telephones the undersigned to discuss this before the Rule 71(3) Communication is issued. I look forward to hearing from the Examiner in due course.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

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15 August 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT/cxe

Thank you for the Summons to Oral Proceedings dated 7 May 2018. Our further submissions on the points raised by the Examining Division in the Summons are set out below. We attach the following documents:

- a second Declaration of Dr Bruce Andrien dated 15 August 2018;
- an extract from Alberts et al (1994);
- a copy of the Opposition statement filed against EP1720571 (D11);
- a redacted version of the representative clinical trial protocol C04-001; and
- a redacted version of the representative clinical trial protocol C02-002.

The comments below address each and every issue identified by the Examining Division in the Summons to Oral Proceedings. Given the early filing of this response in advance of the 5 February 2019 date set for Oral Proceedings, the Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback, so that the Applicant has time to address any outstanding issues before the date set for filing final written submissions.

Articles 76(1) and 123(2) EPC

The amendment complies with Rule 139 EPC because a skilled person would have immediately recognised that the applicant intended to claim only the mature antibody.

The amendment of the description and claims to refer to an antibody having a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 and to an antibody having a light chain variable region consisting of residues 23-214 of SEQ ID NO: 4 meets the requirements of Articles 76(1) and 123(2) EPC.

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The Examining Division considers that this amendment is not a correction that complies with Rule 139 EPC because

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application as originally filed were actually those for which protection was sought."*

However, the skilled person would have immediately recognised that the applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> *"The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1; emphasis added)*

It was common knowledge that immature heavy and light chains included a signal peptide at the N-terminus, and a skilled person could have readily identified the signal peptide in SEQ ID NOs: 3 and 4.

As addressed in the Andrien Declaration filed on 10 July 2017, it was common general knowledge in the art at the filing date of the application that a signal peptide is present at the N-terminus of immature heavy and light chain polypeptides. Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) (Exhibits B to E filed with the Andrien Declaration on 10 July 2017), which is a database of biological sequences containing sequence alignments, is evidence of the skilled person's common general knowledge and shows the signal peptides of various human light chains.

Page 1 from Kabat *et al.* shows the 22-residue signal peptides of human kappa light chains. The first three residues are predominantly Met Asp Met and the last two residues are predominantly Arg Cys. In each of SEQ ID NO: 3 and SEQ ID NO: 4 of the patent, the first three residues are Met Asp Met and residues 21 and 22 are Arg Cys. Therefore, the skilled person would recognise that SEQ ID NO: 3 and SEQ ID NO: 4 are the amino acid sequences of the immature light chain variable region and the light chain, respectively, that contain a signal peptide that is not present in the mature antibody.

Furthermore, the skilled person would know from his common general knowledge that the framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat *et al.* Residues 23

to 26 of SEQ ID NO: 4 are Asp Ile Gln Met, which the skilled person would recognise as being the start of the mature light chain of the disclosed antibody.

It is also part of the common general knowledge of the skilled person that antibodies are produced using cell-based recombinant methods, and not by artificially synthesising the polypeptide chains. The attached second Declaration of Dr Bruce Andrien dated 15 August 2018 refers to the textbook Alberts et al (1994) Molecular Biology of the Cell and explains that antibodies are processed by the producing-cell in the same way as other secreted proteins, and that this processing always results in the N-terminal signal peptide being cleaved off such that it is not present in the mature antibody.

Thus, during antibody production, the light chain polypeptide sequence of SEQ ID NO: 4, or of SEQ ID NO: 3, is processed to remove the signal peptide. Therefore, the skilled person would know based on his common general knowledge that it would not have been the intention of the Applicant to seek protection for an antibody that contains a signal peptide.

Accordingly, the correction to refer to an antibody comprising only the portion of SEQ ID NO: 3 or SEQ ID NO: 4 that reflects the mature antibody is an obvious correction in the sense that it is immediately evident that nothing else would have been intended than what is offered as the correction within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the application as filed.

The corrections being made to page 5 of the description to refer to the portion of the light chain variable region or the light chain that would be found in the antibody would therefore immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC, Article 76(1) EPC and Article 123(2) EPC.

It follows that claim 1, which refers only to the portion of the light chain sequence shown in SEQ ID NO: 4 that is actually found in the mature antibody, also complies with the requirements of Article 76(1) EPC and Article 123(2) EPC.

The EPO has routinely accepted corrections of this exact type.

The European Patent Office has on other cases accepted that correction of applications defining an antibody as comprising heavy and light chain sequences including signal peptides to redefine the antibody as comprising only the mature proteins of the heavy and light chain sequences meets with the requirements of Rule 139 EPC. Two such examples include EP1558648, in which the correction allowed by the Examining Division was unsuccessfully challenged by the Opponent in an Opposition, and EP2581113.

Article 54 EPC

Eculizumab was not available to the public before the filing date of the present application.

The Examining Division has, after considering the arguments provided in relation to the question of whether eculizumab was publicly available at the filing date of the present application, expressed its opinion in paragraphs 1 to 4 of the Summons. Our comments on each of these points are set out below.

1. The fact that Alexion Pharmaceuticals received marketing authorisation for eculizumab from the FDA on 16 March 2007 means that eculizumab was not available on the market at the 15 March 2007 filing date of the present application. This appears to be accepted by the Examining Division. The Examining Division considers, however, that the antibody could still have been available to the public or that its structure could have been known before the priority/filing date of the present application. As discussed below, eculizumab was not publicly available and its structure was not publicly known at the filing date.

2. It is clear from Mr Saxe's Declaration that Alexion Pharmaceuticals inadvertently submitted the incorrect sequence of eculizumab to the Chemical Abstract Services (CAS) such that the sequence entered into the CAS STN database on 14 February 1999 did not contain the heavy and light chain sequences set out in claim 1 of the present application. It is irrelevant whether or not Alexion had any intention of keeping the substance secret after the date of submission to CAS. It is similarly also irrelevant whether anybody to whom the erroneous sequence in CAS would have been obvious could have contacted Alexion Pharmaceuticals to inquire about the correct sequence. Although Mr Saxe corrected the sequence submitted to CAS as soon as he became aware of the error, the fact remains that as of the filing date of the present application, the correct sequence of eculizumab was not available via CAS.

   It is completely alien to the concept of novelty at the EPO that a mistake in a document, the correction of which is not immediately obvious, can be treated as if the document disclosed the correct information. The fact is that the correct sequence was not disclosed in the CAS registry on 14 February 1999. What was *presumably intended to be disclosed* in the CAS registry on 14 February 1999 does not matter for purposes of assessing novelty.

   Not only was an incorrect sequence disclosed on 14 February 1999, but the corrections that would need to be made to the sequences entered on the CAS STN database on 14 February 1999 to arrive at the correct sequence of eculizumab as disclosed in SEQ ID NOs: 2 and 4 of the present application would not have been immediately obvious to the skilled person.

   Firstly, with regard to the light chain sequence, there was a single error in the sequence of the light chain that was submitted in 1999 as explained in the letter of 28 April 2009 from Dr Stephen Saxe to CAS, which letter was enclosed with the Saxe Declaration filed with our response of 16 January 2018. This error was the inclusion of an arginine (R) residue at position 38 of the light chain sequence instead of a glutamine (Q) residue at this position. It would not have been obvious to the skilled person that the inclusion of an arginine residue instead of a glutamine residue was an error. Indeed, as

shown in Figure 1B at page 1392 of D7, the 5G1.1 and h5G1.1 antibodies referred to therein contain an arginine (R) at position 38.

With regard to the heavy chain amino acid sequence, the sequence originally submitted and included in the STN database on 14 February 1999 was a 650 amino acid sequence, whereas the correct sequence has a length of only 448 amino acids. As shown in the annotated version of the incorrect heavy chain sequence originally submitted to CAS, which is included with the Saxe Declaration, the initially submitted sequence contains three blocks of amino acid residues that are not present in the eculizumab sequence. The first two blocks of amino acid sequences are inserted and the third block of amino acids substitutes a shorter stretch of amino acids present in eculizumab but missing in the incorrect sequence originally submitted to CAS. In addition, there are two single amino acids in the constant region of the heavy chain sequence that differ between the originally submitted sequence and the correct sequence. All of the errors in the originally submitted sequence would not have been obvious to the skilled person.

Even if the skilled person had recognised that the heavy chain sequence in the CAS STN database was unusually long for a heavy chain sequence and had turned to Thomas *et al*, 1996 (D7 in the present proceedings), which is the literature paper describing an antibody referred to as h5G1.1, he would have seen that the human IgG4 isotype was chosen to prepare a full length humanised 5G1.1 antibody in D7 (see D7, page 1396, sentence spanning left and right columns and page 1399, right column, lines 24 to 31). The skilled person would not have known that the heavy chain constant region intended to be submitted to CAS is not the IgG4 constant region used in D7.

The amino acid sequence of the IgG4 heavy chain in D7 differs from the heavy chain shown in SEQ ID NO: 2 of the present application and as correctly submitted to CAS in April 2009. The differences are illustrated at pages 2 to 3 of the Declaration of Dr Leonard Bell filed on 10 July 2017. There are 14 amino acid residues that differ between the IgG4 heavy chain constant region sequence and the heavy chain constant region sequence of eculizumab shown in SEQ ID NO: 2 of the present application. As shown in the Bell Declaration, there are four differences in the CH1 domain, six differences in the hinge region and four differences in the CH2 domain. Therefore, even if the skilled person would have turned to D7 to try and determine the correct sequence of eculizumab, D7 would have provided him with a very different sequence than that described in SEQ ID NO: 2.

In the third party observations filed 12 December 2017, attention was drawn to a statement made by the Patentee in the Opposition proceedings against EP1720571 in response to an allegation by the Opponent that the claims of that patent were insufficiently disclosed. A copy of the Opposition statement filed by Pfizer Inc against EP1720571 is attached as D11. It can be seen from Section 7.1 at pages 12 to 13 of this Opposition statement that the Opponent alleged that the complete sequence of eculizumab is not disclosed in U.S. Patent No. 6,355,245 or in Thomas *et al*, *Molecular Immunology*, Vol.33, No.17/18, pages 1389-1401, 1996 (D7 in the present proceedings).

In response to this objection, in paragraph 5.1.2 of the response filed on 30 December 2013, the Patentee stated that:

> *"The Patentee asserts that the sequence for eculizumab was publically available prior to the priority and filing date. A sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry No. 219685-560-4"* [emphasis added].

The copy of the CAS entry No. 219685-50-4 that was attached with the response of 30 December 2013 did not include any sequence information. Only a one page fax dated 04/08/2008 (8 April 2008) from CAS was filed with the response (a copy of which was provided with the third party observations filed on 12 December 2017). As the sequence of eculizumab on file at CAS had not been corrected as of 8 April 2008, and certainly not as of the priority date of the opposed patent, this statement by the Patentee referenced an incorrect sequence of eculizumab and was clearly made in error. It is clear that this erroneous statement was made inadvertently and in good faith. Neither the third party observer nor the Examining Division has pointed to any evidence of bad faith in conjunction with the incorrect statement, and indeed none exists. Further, despite this unfortunate mistaken statement in the Opposition response, the fact remains that the full correct sequence of eculizumab was not publicly disclosed or publicly available before 16 March 2007.

It is also noted that Alexion's statement in the aforementioned opposition response was never challenged because the Opponent had withdrawn the Opposition on 7 October 2013. Nor was Alexion called on by the Opposition Division to provide evidence of the eculizumab sequences published by CAS in 1999. Indeed, sufficiency of disclosure was not further discussed in the Opposition proceedings on EP1720571. The assertion made by Alexion was not relied on in any way in those proceedings, and the statement had no influence on the outcome of the proceedings, in which EP1720571 was revoked for lack of inventive step. Thus, Alexion's mistaken statement amounted to nothing more than a mere assertion that turned out to be erroneous but that had no bearing on the outcome of that case. Alexion obtained no benefit as a result of the inadvertent misstatement.

The third party argued in paragraph I.3 of his observations of 12 December 2017 that:

> *"in light of Alexion's past actions, Alexion's declarations in the present case have to be considered to contravene against the generally accepted legal principle of good faith. In particular, in this case, the principle of venire contra factum proprium makes these declarations inadmissible".*

However, the legal principle of *nemo potest venire contra factum proprium* does not apply in this situation.

The principle of *nemo potest venire contra factum proprium* has been applied by the EPO's legal Boards of Appeal where the EPO has exhibited contradictory behaviour, i.e. where the actions of the EPO have given rise to legitimate expectations which precluded the EPO from later going back on its own previous conduct (see for example, J27/94 and J14/94 summarised in Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 519, paragraph 2.3).

In the present case, the assertion made in the Opposition response was not relied on or considered any further in the Opposition proceedings. It did not affect the outcome of the Opposition proceedings in which the patent was revoked for lack of inventive step. Accordingly, the assertion made in the response to the opposition of EP1720571 does not take precedence over the signed declarations made in connection with the present application, and it does not bind Alexion Pharmaceuticals in any way.

The Examining Division considers that:

> *"Even if it were now purported that Alexion Pharmaceuticals would have never disclosed the correct sequence to anybody who had asked them for it, that would not amount to a valid defence since under the legal principle of "nemo potest venire contra factum proprium" Alexion Pharmaceuticals would be bound to its declared intention to lay the compound open under CAS rules in order to have other patents be regarded as sufficiently disclosed".*

It appears that the Examining Division is attempting to apply the legal principle of *nemo potest venire contra factum proprium* in a different way than that suggested by the third party. Whilst the third party cited this legal principle in order to suggest that all of the declarations filed in the present application should be ignored, the Examining Division appears to consider that this principle would have bound Alexion Pharmaceuticals to disclose the correct sequence of eculizumab to anybody who requested it in order to have "*other patents be regarded as sufficiently disclosed*". It is presumed that the Examining Division is using the term "*other patents*" to refer to the patents and applications listed in D8 filed by the third party.

There are a number of points to be made here. Firstly, the legal principle of *nemo potest venire contra factum proprium* does not oblige a party who has submitted incorrect data to provide the correct data to any third party who requests it. Alexion is also unaware of what "CAS rules" the Examining Division refers to.

Secondly, the Examining Division seems to suggest that even though, in fact, the correct sequence of eculizumab was not disclosed before 16 March 2007, it must be considered to have been effectively disclosed so that the antibody defined using the term "eculizumab" used in previously-filed patents and patent applications listed in D8 is sufficiently disclosed. Clearly, this reasoning is legally flawed. Lack of novelty cannot be established based on an unsupported presumption of sufficiency of an earlier patent. Rather, the issue of novelty solely rests on the factual inquiry of whether or not the

claimed invention was in the public domain prior to the effective filing date. In the present case, it was not. Therefore, the claims are novel.

Moreover, all of the patents and patent applications listed in D8, with the exception of EP1720571 which was revoked for lack of inventive step, refer to eculizumab by name only in dependent claims (see the right column in the Table in D8). The independent claims of these patents and applications refer to anti-C5 antibodies more generally.

There is no doubt that anti-C5 antibodies were sufficiently disclosed in 2004, which is the year of the earliest priority date of any of the patents and patent applications listed in D8. For example, all of the patents and patent applications refer to US Patent 6,355,245 and many refer also to Thomas *et al*, 1996 (D7 in the present proceedings). These documents both disclose heavy and light chain variable region sequences of anti-C5 antibodies, and D7 also discloses a full length humanised IgG4 anti-C5 antibody.

In any event, as stated above, it is of no import whether disclosure of the full correct sequence of eculizumab would have been necessary in order for all of the claims of the earlier patents and patent applications to meet the sufficiency requirements of Article 83 EPC - the *fact* remains that the full correct sequence of eculizumab was *not* disclosed prior to 16 March 2007.

The Examining Division considers that:

> "*The statement from the Applicant acting as Patentee on EP1720571 (see D9, page 14 Item 5.1.2) confirms the fact that he considered the structure of eculizumab to have been disclosed at the filing date of the present application*".

Again, this statement from the Patentee was an inadvertent error made in good faith. The issue of sufficiency was not further discussed in the course of the opposition and the assertion made by the Patentee was not challenged, the Opponent having withdrawn his Opposition on 7 October 2013. In the statement made in connection with the Opposition against EP1720571, Alexion Pharmaceuticals made no reference to SEQ ID NO: 2 or SEQ ID NO: 4, and did not make any admission that these particular sequences were publically available.

Also, the fact noted by the Examining Division that the date of the assertion in D9 (30 December 2013) is later than the date in which the correction of the eculizumab structure was requested to CAS is irrelevant to the discussion of novelty of the present application. The assertion cannot alter what was, or was not, disclosed at an earlier date.

The fact remains that the sequences submitted by Alexion Pharmaceuticals to CAS and entered into the CAS STN database on 14 February 1999 are not the sequences of SEQ ID NOs: 2 and 4 set out in claim 1 of the present application.

3. The Examining Division considers that the

> *"many patent and non-patent literature documents published before the priority date and relating to the use of eculizumab can be regarded as evidence that the compound was well-known and available to the public before the priority date of the patent application".*

However, this position is not in line with the case law. The Boards of Appeal have established that the mention of a compound in a published document does not mean that the compound is available to the public.

In T576/91, the Board of Appeal stated that if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "*available to the public*" within the meaning of Article 54(2) EPC.

Further, in T128/92 the Board of Appeal stated that for a complex biochemical to be made available to the public, the minimum that would seem to be required for publication was a notice to those in the field that samples of the biochemical could be obtained upon request, and clear evidence of exactly what the biochemical was. (See Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 89, paragraph 3.2.5).

At page 2 paragraph 2.1(a) of the Summons, the Examining Division expresses the opinion that

> *"since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the products had not be available for public use or that the working group using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals, it had to be concluded that the structure of the antibody was available to the public at the time of filing of the present application".*

This conclusion is, however, clearly contrary to the case law discussed above. It cannot be concluded from a publication that an antibody was publicly available based merely on the absence of a statement that the antibody was *not* available. To the contrary, a positive statement to this effect would be required. No such statement is included in any of the cited documents.

The Examining Division has noted that different published patent applications belonging to the Applicant that mention "eculizumab" were available at the priority date of the present application. Such patents and applications are listed in D8 filed by the third party on 12 December 2017. None of these earlier applications, or the references they cite, disclose the full sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

The mere existence of earlier patents and applications that mention "eculizumab" without precisely defining it does not destroy the novelty of

claim 1 of the present application. The present Examining Division is tasked with assessing the novelty of the claims of the present application in light of all of the *factual* information that was available in the prior art, and the fact is that none of the patents and applications listed in D8 disclose an antibody comprising the full heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

4. The Examining Division has requested proof of the confidentiality agreement between the company and each participant/physician involved in the studies described in D1 to D5. Dr Laural Boone, a Senior Director and Patent Counsel at Alexion, stated that she has reviewed the protocols of all of the clinical trials that were carried out with eculizumab prior to 16 March 2007 and found strict confidentiality and drug accountability provisions in each of the protocols. (See the Declaration of Dr Boone filed on 10 July 2017).

The Table at page 3 of Dr Boone's declaration lists all of the trials for which she reviewed the protocols, and paragraphs 7 and 8 of her Declaration provide examples of the confidentiality provisions she found in the protocols. The representative protocols from the studies Dr Boone refers to, C04-001 and C02-002, are now attached in redacted form. The confidentiality provision referred to in paragraph 7 of the Boone Declaration is found in paragraph 11.1 of protocol number C04-001, and the provision referred to in paragraph 8 of the Boone Declaration is found in the final paragraph at page 46 of study number C02-002.

In addition, as paragraph 10 of Dr Boone's declaration makes clear, the protocols also contained strict drug accountability provisions. The provisions referred to in paragraph 10 of Dr Boone's declaration are set out in paragraph 4.6 spanning pages 19 and 20 of protocol number C04-001, and in paragraph 6.6 at page 24 of study number C02-002. These provisions ensured that no study drug (eculizumab) was released to the public, and, as stated in the Boone Declaration, were included in all clinical trial protocols identified in the Declaration. This evidence demonstrates that all physicians and participants in the clinical trials were bound by strict confidentiality and drug accountability provisions so that there was no public disclosure or public availability of eculizumab via the trials.

Moreover, it should be noted that even if the participants and physicians involved in the studies had not had these strict conditions imposed on them by their agreements with Alexion Pharmaceuticals, the structure of eculizumab still would not have been publicly available, because, in accordance with G1/92, the skilled person would not have been able to analyse the structure of eculizumab and reproduce it without an undue burden.

In T2045/09 the Board considered whether the public sale of an antibody made that antibody publicly available in the sense that the skilled person would be able to determine its internal structure and reproduce it without unburden in accordance with G1/92.

In T2045/09, the Board noted that in a document published in 2006, the Authors emphasised that the sequencing of the variable regions of antibodies comprising the CDRs constituted a major scientific challenge and that:

> "*In particular, the unique character of the CDRs renders an alignment and the comparison of the data to already existing antibody sequences in databases impossible. A complete and sufficiently correct amino acid sequence could only be achieved by complementing the data of the Edman sequencing with sequence obtained independently from mass spectrometry*".

In view of the challenge the skilled person would have faced in trying to sequence an antibody in 2006, the Board was not persuaded that the skilled person could have correctly determined the amino acid sequence of the relevant antibody with the knowledge and the technologies available (see Reasons 35 and 36 of the Decision).

In the Summons at page 2, last paragraph, the Examining Division points to the official Communication dated 17 November 2017 in which it

> "*pointed out that any of the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. Reference was also made to D7, which disclosed most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explained how the sequence was found*".

However, at least the uniquely-engineered, non-naturally-occurring portions of eculizumab were not disclosed in D7 and, as explained above as found in T0245/09, obtaining the full sequence of eculizumab from the eculizumab molecule alone would not have been routine in the art at the 15 March 2006 priority date of the present application.

2.2 The Examining Division maintains that the disclosure of D1-D5 teaching eculizumab and its use in the treatment of PNH are considered novelty destroying for the subject matter of the claimed invention. The claims of the present application are, however, novel in view of the disclosure of D1-D5.

Each of D1-D5 refers to eculizumab by name only. None of these documents disclose the heavy and light chain sequences of eculizumab. As discussed in paragraphs 2.1(3) above, the mere mention of a complex biochemical by name in a publication does not enable that complex biochemical to be made or make that complex biochemical available to the public. Therefore, the claims of the patent application are novel over D1-D5.

2.3 The present application is novel over the disclosure of D10. D10 is an earlier patent application of Alexion Pharmaceuticals (WO 2005/074607). This document does not provide an enabling disclosure for an antibody having the heavy and light chain sequences of SEQ ID NOs: 2 and 4.

WO 2005/074607 relates to methods of treating a hemolytic disease by administrating an anti-C5 antibody to a subject having a hemolytic disease (see claim 1 of D10). D10 discloses that:

> *"Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanised Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol.33, No.17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab"* (page 18, lines 12 to 20).

Thus, D10 defines eculizumab by reference to U.S. Patent No. 6,355,245 and Thomas *et al*, 1996 (D7 in the present proceedings). Neither of these documents discloses the sequences of SEQ ID NOs: 2 and 4. Therefore an antibody having the heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4 is novel over D10.

The assertion made by the Applicant as Patentee in the response to the Opposition against EP1720571 derived from D10 cannot change what is disclosed in D10, and, as discussed above, is not an admission that SEQ ID NO:2 and SEQ ID NO: 4 were publically known at the filing date of EP1720571. The fact is that D10 does not disclose an antibody having the mature heavy and light chain sequences of SEQ ID NOs: 2 and 4, and nothing said later can change this. Therefore, the claims of the present application are novel over D10.

In conclusion, none of the prior art documents disclose an antibody having the sequences recited in claim 1, and such an antibody was not publically available in the sense of Article 54(2) EPC. Therefore, claim 1 is novel.

Article 56 EPC

The claims of the present application are novel for the reasons set out above. The claims also involve an inventive step in accordance with Article 56 EPC.

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive.

SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human

IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and it would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any further information about the structure of eculizumab. It would therefore not have been obvious to the skilled person from any of these documents to engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2.

Accordingly, claim 1 involves an inventive step.

Article 83 EPC

Regardless of the conclusions reached with respect to Articles 76(1) EPC, Article 123(2) EPC and Article 54 EPC, the claimed subject matter is sufficiently disclosed in the present application.

Claim 1 is directed to:

> "*An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4.*"

It would be within the routine skill of a person skilled in the art to make an antibody having the specified heavy and light chain amino acid sequences.

There is also no doubt that the sequences disclosed in the present application identify the structure of eculizumab. SEQ ID NO: 2 is the amino acid sequence of the full mature heavy chain of eculizumab and the sequence of SEQ ID NO: 4 is the amino acid sequence of the full immature light chain of eculizumab. Therefore, the sequences filed with the original application are those identifying the structure of eculizumab.

For the reasons explained above, the skilled person would know based on his common general knowledge which residues of SEQ ID NO: 4, or of SEQ ID NO: 3, correspond to the signal peptide and which residues correspond to the mature portion of the therapeutic antibody known as eculizumab, i.e. residues 23 to 214 of SEQ ID NO: 4.

Furthermore, in terms of actually producing the eculizumab antibody, a polynucleotide encoding the whole of SEQ ID NO: 4 could be expressed in a cell along with a polynucleotide encoding the heavy chain of eculizumab, i.e. SEQ ID NO: 2. The signal peptide of SEQ ID NO: 4 would be cleaved off during antibody production in the cell, resulting in an antibody whose light chain contains only the mature portion of SEQ ID NO: 4.

Thus, the skilled person would have known from the disclosure of the present application which structure was to be assigned to the therapeutic antibody eculizumab.

Conclusion

The Examining Division is requested to reconsider this application favourably. The Applicant has addressed each and every issue identified by the Examining Division in the Summons to Oral Proceedings. Given the early filing of this response in advance of the 5 February 2019 date set for Oral Proceedings, the Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback. It is hoped that the application can be allowed without the need for Oral Proceedings. Accordingly, the Examiner is requested to telephone the undersigned once the Examining Division has considered our submissions.

I look forward to hearing from the Examiner.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

AUXILIARY~~MAIN~~ REQUEST 2

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells ~~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.

2. An antibody according to claim 1, wherein the antibody is obtainable by production in transfected CHO or NS0 cells.

3. A pharmaceutical composition comprising the antibody of claim 1 or claim 2.

Samsung Bioepis Exhibit No. 1028
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AUXILIARY REQUEST ~~2~~8

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. An antibody for use according to claim 1, wherein the transfected cells are CHO or NS0 cells.

3. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1 or claim 2~~.

4. A pharmaceutical composition for use according to claim 3, wherein the transfected cells are CHO or NS0 cells.

Samsung Bioepis Exhibit No. 1028
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AUXILIARY REQUEST ~~1~~7

CLAIMS

1. An antibody that binds C5, wherein the antibody is eculizumab and comprises a heavy chain consisting of SEQ ID NO: 2, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5, wherein the antibody is eculizumab and comprises a heavy chain consisting of SEQ ID NO: 2, for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

Samsung Bioepis Exhibit No. 1028
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AUXILIARY REQUEST ~~3~~9

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected CHO or NS0 cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising ~~the~~ an antibody that binds C5, wherein the antibody is obtainable by producing in transfected cells a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

Samsung Bioepis Exhibit No. 1028
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AUXILIARY~~MAIN~~ REQUEST 4

CLAIMS

1. An antibody that binds C5 comprising the ~~a~~ heavy chain amino acid sequence shown in ~~consisting of~~ SEQ ID NO: 2 and the~~a~~ light chain amino acid sequence shown in ~~consisting of residues 23 to 214 of~~ SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

Samsung Bioepis Exhibit No. 1028
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AUXILIARY~~MAIN~~ REQUEST 6

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

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~~MAIN~~ AUXILIARY REQUEST 1

CLAIMS

1. An antibody that binds C5, wherein the antibody is eculizumab and comprises ~~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~~.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY~~MAIN~~ REQUEST 5

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of ~~residues 23 to 214 of~~ SEQ ID NO: 4.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST 410

CLAIMS

1. An antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5 comprising the heavy chain amino acid sequence shown in SEQ ID NO: 2 and the light chain amino acid sequence shown in SEQ ID NO: 4 for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

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AUXILIARY~~MAIN~~ REQUEST 3

CLAIMS

1. An antibody that binds C5, wherein the antibody is obtainable by producing in transfected CHO or NS0 cells ~~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.

2. A pharmaceutical composition comprising the antibody of claim 1.

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AUXILIARY REQUEST ~~5~~11

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria.

2. A pharmaceutical composition comprising an ~~the~~ antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4, for use in treating paroxysmal nocturnal hemoglobinuria~~of claim 1~~.

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Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	8743931
Application number	EP16195355.9
Date of receipt	19 June 2020
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file

Documents submitted

package-data.xml	ep-sfd-request.xml
epf1038.pdf (2 p.)	APPEAL-GRDS-1.PDF\Statement of Grounds of Appeal N411599EP-B.PDF (34 p.)
APPEAL-LETT-6.PDF\Auxiliary Request 1 (clean) N411599EP-B.PDF (1 p.)	APPEAL-LETT-7.PDF\Auxiliary Request 1 (tracked) N411599EP-B.PDF (1 p.)
APPEAL-LETT-8.PDF\Auxiliary Request 2 (clean) N411599EP-B.PDF (1 p.)	APPEAL-LETT-9.PDF\Auxiliary Request 2 (tracked) N411599EP-B.PDF (1 p.)
APPEAL-LETT-10.PDF\Auxiliary Request 3 (clean) N411599EP-B.PDF (1 p.)	APPEAL-LETT-11.PDF\Auxiliary Request 3 (tracked) N411599EP-B.PDF (1 p.)
APPEAL-LETT-12.PDF\Auxiliary Request 4 (clean) N411599EP-B.PDF (1 p.)	APPEAL-LETT-13.PDF\Auxiliary Request 4 (tracked) N411599EP-B.PDF (1 p.)
APPEAL-LETT-14.PDF\Auxiliary Request 5 (clean) N411599EP-B.PDF (1 p.)	APPEAL-LETT-15.PDF\Auxiliary Request 5 (tracked) N411599EP-B.PDF (1 p.)
APPEAL-LETT-16.PDF\Auxiliary Request 6 (clean) N411599EP-B.PDF	APPEAL-LETT-17.PDF\Auxiliary Request 6 (tracked)

(1 p.) APPEAL-LETT-18.PDF\Auxiliary Request 7 (clean) N411599EP-B.PDF (1 p.) APPEAL-LETT-20.PDF\Auxiliary Request 8 (clean) N411599EP-B PXT.PDF (1 p.) APPEAL-LETT-22.PDF\Auxiliary Request 9 (clean) N411599EP-B.PDF (1 p.) APPEAL-LETT-24.PDF\Auxiliary Request 10 (clean) N411599EP-B.PDF (1 p.) APPEAL-LETT-26.PDF\Auxiliary Request 11 (clean) N411599EP-B.PDF (1 p.) APPEAL-LETT-1.PDF\Main Request N411599EP-B.PDF (1 p.) APPEAL-ANNEX-2.pdf\Annex 2 - Submissions of 15 Aug 2018 N411599EP-B.pdf (14 p.) APPEAL-LETT-5.pdf\D32 - Third Declaration of Andrien.pdf (5 p.) APPEAL-LETT-3.PDF\D34 Declaration of Webb.PDF (6 p.) APPEAL-LETT-28.PDF\File Grounds of Appeal N411599EP-B PXT.PDF (1 p.)	N411599EP-B.PDF (1 p.) APPEAL-LETT-19.PDF\Auxiliary Request 7 (tracked) N411599EP-B.PDF (1 p.) APPEAL-LETT-21.PDF\Auxiliary Request 8 (tracked) N411599EP-B PXT.PDF (1 p.) APPEAL-LETT-23.PDF\Auxiliary Request 9 (tracked) N411599EP-B.PDF (1 p.) APPEAL-LETT-25.PDF\Auxiliary Request 10 (tracked) N411599EP-B.PDF (1 p.) APPEAL-LETT-27.PDF\Auxiliary Request 11 (tracked) N411599EP-B.PDF (1 p.) APPEAL-ANNEX-1.PDF\Annex 1 - Submissions of 5 July 2019 N411599EP-B.PDF (18 p.) APPEAL-ANNEX-3.PDF\Annex 3 - Submissions of 16 January 2018 N411599EP-B.PDF (8 p.) APPEAL-LETT-4.PDF\D33 Laffly and Sodoyer 2005.PDF (24 p.) APPEAL-LETT-2.pdf\D35 - Declaration of Hill.pdf (2 p.)

Submitted by	CN=Pamela Tuxworth 23470
Method of submission	Online
Date and time receipt generated	19 June 2020, 18:01 (CEST)
Message Digest	C9:6B:2E:3F:D7:DE:96:FB:43:B1:DD:4D:28:71:2F:B0:06:85:65:65

Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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J A Kemp LLP
14 South Square
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London WC1R 5JJ
ROYAUME UNI

Datum/Date
06.07.20

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
APPR	16195355.9 / 3167888
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
Alexion Pharmaceuticals, Inc.	

File number: **T1515/20-3.3.01**

Commencement of proceedings before the Board of Appeal

The letter dated 24.03.20 filed by the applicant
against the decision of the European Patent Office of 11.02.20 .
has been referred to Board of Appeal 3301.

The reference number is mentioned above.

Any further communications should be addressed to the Boards of Appeal of the European Patent Office and should quote this reference number.

The Registrar M. Schalow
Tel.: 089 / 2399 - 3311

Annexe(s): copy of the notice of appeal for the other party(ies)
Practical instructions

Registered letter

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Page 1594

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Fax +49 (0)89 2399-4465

File number: **T1515/20-3.3.01**

Practical instructions for parties to appeal proceedings

Submissions should preferably be filed electronically using either EPO online filing (OLF) or the EPO case management system (CMS). Please note that Web-Form Filing is not permitted in appeal proceedings (see Decision of the President of the EPO dated 9 May 2018 concerning the electronic filing of documents (OJ EPO 2018, A45)).

Please annex all documents, if possible with a list of complete references, and number them in a sequence that is consistent with the numbering used in the first-instance proceedings and, if appropriate, the numbering used by other parties.

Please also forward copies of submissions to all other parties to the appeal proceedings direct, prior to the date of the oral proceedings.

Your attention is drawn to Rule 103 EPC, which provides for reimbursement of the appeal fee in certain cases if the appeal is withdrawn. For the version in force from 1 April 2020, see OJ EPO 2020, A5.

Your attention is further drawn to the provisions of the new Rules of Procedure of the Boards of Appeal (in force from 1 January 2020 - RPBA 2020, see OJ EPO 2019, A63), in particular Articles 12, 13, 15 and 16 RPBA 2020.

For more information on the Boards of Appeal, please consult the Boards of Appeal web page, to be found on the EPO website under "Boards of Appeal".

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Page 1595

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Fax +49 (0)89 2399-4465

Appeal number: T1515/20-3.3.01

Order

1. In accordance with the business distribution scheme of the Technical Boards of Appeal, the following shall hear the above appeal:

Chairman: A. Lindner

technically qualified member: M. Pregetter

as legally qualified member: L. Bühler

In the case of an extended Board:

technically qualified member: _____

as legally qualified member: _____

2. The rapporteur shall be: M. Pregetter

3. The additional rapporteur shall be: _____

4. Back to the Registry for further action.

Munich, 23.04.21

The Chairman: A. Lindner

Europäisches Patentamt
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Request for changes relating to an association of professional representatives

European Patent Office
Directorate 5.2.3 – Legal Division
80298 Munich
GERMANY

Name of association

J A Kemp LLP

Registration No.

811

Address

14 South Square
Gray's Inn
GB-LONDON WC1R 5JJ

I. Request for a change in the composition

☐ We request registration of a change in the composition of the association with effect from the date of receipt of the present request at the EPO or from the following subsequent date

Date

Members to be added[1] (see additional sheet where necessary)

Surname	*First name*	*Representative No.*	*Signature*	*Date*

Members to be deleted[1] (see additional sheet where necessary)

Surname	*First name*	*Representative No.*	*Signature*	*Date*

☒ Confirmation by at least one member of the association (see additional sheet where necessary)

Surname	*First name*	*Representative No.*	*Signature*	*Date*

[1] This form is only valid for changes relating to an association. If the address in the list of professional representatives before the European Patent Office (see Article 134(1) EPC) should also be changed, a separate request using EPO Form 52301 (Request for registration of changes relating to an entry in the list of professional representatives before the European Patent Office) should be filed.

EPO 52305 02.16

II. Request for a change of name[1]

☐ We request registration of a change of name of the association with effect from the date of receipt of the present request at the EPO or from the following subsequent date

Date

The new name is

☒ Confirmation by at least one member of the association (see additional sheet where necessary)

Surname	*First name*	*Representative No.*	*Signature*	*Date*

III. Request for a change of address[1]

☑ We request registration of a change of address of the association with effect from the date of receipt of the present request at the EPO or from the following subsequent date

Date 27.9.21

The new address is

80 Turnmill Street
GB-LONDON EC1M 5QU

☒ Confirmation by at least one member of the association (see additional sheet where necessary)

Surname	*First name*	*Representative No.*	*Signature*	*Date*
Price	Nigel John King	62100		20.9.21

IV. Number of annexes (e.g. additional sheet for further members)

[1] This form is only valid for changes relating to an association. If the address in the list of professional representatives before the European Patent Office (see Article 134(1) EPC) should also be changed, a separate request using EPO Form 52301 (Request for registration of changes relating to an entry in the list of professional representatives before the European Patent Office) should be filed.

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Fax +49 (0)89 2399-4465

Appeal number: T1515/20-3.3.07

Order

Change of the composition of the Board

compare to previous order

1.

Chairman: A. Usuelli

technically qualified member: J. Molina de Alba

technically qualified member: J. Lécaillon

as legally qualified member: L. Bühler

as legally qualified member: L. Basterreix

2. The rapporteur shall be: J. Molina de Alba

3. The additional rapporteur shall be: _____

4. Reasons:
Change in the business distribution scheme of the board for the current year.

5. Back to the Registry for further action.

Munich, 15.07.22

A. Usuelli

EPO Form 3303.16

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Appeal number: T1515/20-3.3.07

Order

1. The main classification symbols assigned upon filing to European patent / European patent application No. 16195355.9, were A61K31/7105, which means that under the business distribution scheme of the Technical Boards of Appeal for the working year 2022 the competent Board is 3.3.07 .

   However, particular technical content of the appeal make it more appropriate for it to be heard by Board 3.3.04 .

2. The Chairs of the above two Boards have therefore agreed to transfer it from Board 3.3.07 to Board 3.3.04 (Article 1(2) of the business distribution scheme).

Munich 18.07.22

A. Usuelli		M. Pregetter	
Chairman of Board	3.3.07	Chairman of Board	3.3.04

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Appeal number: T1515/20-3.3.04

Order

Change of the composition of the Board

compare to previous order

1.

Chairwoman: M. Pregetter

technically qualified member: B. Claes

technically qualified member: B. Rutz

as legally qualified member: L. Bühler

as legally qualified member: R. Romandini

2. The rapporteur shall be: B. Claes

3. The additional rapporteur shall be: _____

4. Reasons:

5. Back to the Registry for further action.

Munich, 18.07.22

The Chairwoman: M. Pregetter

EPO Form 3303.16

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ROYAUME UNI

Datum/Date
20.07.22

Zeichen/Reference/Référence	
	/ APPR
Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°	16195355.9 / 3167888
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
Alexion Pharmaceuticals, Inc.	

Appeal number: **T1515/20-3.3.01**

Change of responsibility of a Board of Appeal
Article 1(1) Business Distribution Scheme for the Technical Boards of Appeal

According to the amended business distribution scheme for the Technical Boards of Appeal for the year 2022 , this case has been transferred to Board 3.3.07 as from 01.07.22.

The new reference number is: **T1515/20-3.3.07**

Please quote the new reference number cited above in any further communications.

The Registry B. Atienza Vivancos
Tel.: 089 / 2399 - 3371

Registered letter

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80 Turnmill Street
London EC1M 5QU
ROYAUME UNI

Datum/Date
21.07.22

Zeichen/Reference/Référence	
	/ APPR
Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°	16195355.9 / 3167888
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
Alexion Pharmaceuticals, Inc.	

Appeal number: T1515/20-3.3.07

Transfer to another Board of Appeal
Article 1(2) Business Distribution Scheme for the Technical Boards of Appeal

Under the business distribution scheme for the Technical Boards of Appeal, this appeal concerning European application / Patent No. 16195355.9 / 3167888 was allocated to Board 3307 .

However, the technical content of the appeal makes it appropriate for allocation to Board 3.3.04.

The Chairmen of those two Boards have therefore agreed to transfer the appeal to Board 3.3.04 (Article 1(2) of the business distribution scheme).

The new reference number is: T1515/20-3.3.04

Please quote the new reference number cited above in any further communications.

The Registry B. Atienza Vivancos
Tel.: 089 / 2399 - 3371

Registered letter

EPO Form 3056 01/11

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Appeal number: **T1515/20-3.3.04**

Communication of the Board of Appeal pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal

The Rapporteur B. Claes

Beschwerdekammern des Europäischen Patentamts
Boards of Appeal of the European Patent Office
Chambres de recours de l'Office européen des brevets

This document: 2 page(s) including this page

Annex(es):

Communication text

1. This communication accompanies a summons to oral proceedings for **21 and 22 September 2023.** The purpose of this communication is to inform the parties that the board has started the examination on the merits of the appeal.

2. Oral proceedings in this appeal case and in appeal case T 1435/20, both cases being dealt with by the board in the same composition, have now been scheduled for the same dates. This allows that the two cases can be dealt with directly one after the other.

3. The board will in due course issue a further communication pursuant to Article 15(1) RPBA drawing attention to matters that seem to be of particular significance for the decision to be taken and setting out its preliminary opinion, as necessary. The board

Samsung Bioepis Exhibit No. 1028
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will then also inform the appellant with which appeal it intends to start the hearing.

4. It should be noted that this communication does not constitute a communication under Rule 103(2) EPC.

Samsung Bioepis Exhibit No. 1028
Page 1605

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J A Kemp LLP
80 Turnmill Street
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ROYAUME UNI

Date
25.07.22

Zeichen/Reference/Référence	/ APPR
Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°	16195355.9 / 3167888
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	Alexion Pharmaceuticals, Inc.

Appeal number: **T1515/20-3.3.04**

Summons to oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to the oral proceedings concerning the above appeal. The proceedings are scheduled to take place

on 21.09.23 at 09:30 hrs
and on 22.09.23 at 09:30 hrs

Richard-Reitzner-Allee 8, 85540 Haar (DE)

The proceedings will be public.

Upon arrival, please consult the notice board at reception for the number of the room in which the oral proceedings will be held.

A main waiting area is located at the entrance, directly after the security check. Further waiting rooms are located near to the oral proceedings rooms.

You are requested to make your way to the appointed room at least **10 minutes** before the hearing.

Registered letter

Samsung Bioepis Exhibit No. 1028
Page 1606

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Appeal number: **T1515/20-3.3.04**

You are furthermore reminded of the following:

- if a party who has been duly summoned to oral proceedings does not appear, the proceedings may continue without that party (Rule 115(2) EPC).
- oral proceedings will only be postponed at the request of a party for serious reasons (see Article 15(2) of the Rules of Procedure of the Boards of Appeal of the European Patent Office).

- as regards filing authorisations for representatives or company employees, see Decision of the President of the EPO dated 12.7.2007 on the filing of authorisations in Special edition No. 3 OJ EPO 2007, 128.
- concerning the language of the proceedings, attention is drawn to Rule 4 EPC. Notice given pursuant to Rule 4(1) EPC before the first instance is not valid in proceedings before the Boards of Appeal.
- attention is drawn to Rule 103(3)(a), (4)(a) and (c) EPC, according to which it is possible, under certain circumstances, to obtain a partial reimbursement of the appeal fee if the appeal or the request for oral proceedings is withdrawn.

Composition of the Board

The Chairwoman: M. Pregetter
Member: B. Claes
Member: L. Bühler
Member: B. Rutz
Member: R. Romandini

For any urgent communication in connection with the oral proceedings please use only EPO Online Services (preferably) or fax No. + 49 (0) 89 2399 3014.

If you are planning not to attend the oral proceedings or if you are aware of any matter that could have a bearing on the appointment of interpreters, you are requested to inform the Board of this, preferably in writing, at the earliest possible opportunity.

The Registry I. Aperribay
Tel.: 089 / 2399 - 3341

Annex(es):
- [x] Confirmation of receipt Form 3936
- [x] Communication pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal
- [ ] Instructions for mixed-mode oral proceedings
- [ ] Information on technical guidelines Zoom/ EPO data protection guidance

Registered letter

Samsung Bioepis Exhibit No. 1028
Page 1607

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J A Kemp LLP
80 Turnmill Street
London EC1M 5QU
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Boards of Appeal

The Registry

Name: I. Aperribay

Tel.: 089 / 2399 - 3341

Date: 25.07.22

Zeichen/Reference/Référence		/ APPR
Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°	16195355.9	/ 3167888
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire		
Alexion Pharmaceuticals, Inc.		

Do not return if registered letter received via the EPO Mailbox.

Appeal number: T1515/20-3.3.04

EPA/EPO/OEB Formblatt/Form/Formulaire: 3011.1 + 3350

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl. EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately:**

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0)70 340-3016)**
- oder per Post / or by post / ou par courrier

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EPO Form 3936 08/17

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Appeal number: **T1515/20-3.3.04**

Order

Oral proceedings are to be held

on 21.09.23 and 22.09.23

at 09:30 hours

Munich 25.07.22

The Chairwoman:
M. Pregetter

Samsung Bioepis Exhibit No. 1028
Page 1609

J A • K E M P

BY ONLINE FILING

The European Patent Office
80298 Munich
Germany

17 November 2022

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

In accordance with Rule 21(1) EPC, we request rectification of the designation of inventor. A designation of inventor in accordance with Rule 19(1) EPC is attached.

We request that the following inventor is additionally designated on this application:

EVANS, Mark J.
Radnor, Pennsylvania, US

For the avoidance of any doubt, the inventors currently designated are not being replaced.

We look forward to receiving confirmation that the European Patents Register has been updated accordingly.

Electronically Signed
DR PAMELA M TUXWORTH
FOR J A KEMP LLP
ASSOCIATION NO. 811

PATENT ATTORNEYS • TRADE MARK ATTORNEYS
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Our registered office is at 80 Turnmill Street, London EC1M 5QU, UK. Our use of the word 'partner' refers to a member of J A Kemp LLP.

Europäisches Patentamt
European Patent Office
Office européen des brevets

Letter accompanying subsequently filed items

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The document(s) listed below is (are) subsequently filed documents pertaining to the following application:

Application number	16195355.9
Applicant's or representative's reference	N411599EP-B

	Description of document	Original file name	Assigned file name
1	Designation of inventor	Designation of Inventor N411599EP-B.pdf	F1002-1.pdf
2	Documents concerning the inventorship	Additional Inventor N411599EP-B PXT.pdf	INVT-1.pdf

	Payment	
1	Method of payment	Not specified

Signatures

Place:	
Date:	**17 November 2022**
Signed by:	**Elizabeth Davenport 55598**
Association:	**J A Kemp LLP**
Representative name:	**DAVENPORT, Elizabeth**
Capacity:	**(Representative)**

Europäisches Patentamt
European Patent Office
Office européen des brevets

Erfindernennung
Designation of inventor
Désignation de l'inventeur

(falls Anmelder nicht oder nicht allein der Erfinder ist) /
(where the applicant is not the inventor or is not the sole inventor) /
(si le demandeur n'est pas l'inventeur ou l'unique inventeur)

Zeichen des Anmelders / Applicant's reference /
Référence du demandeur

N411599EP-B

Anmeldenummer oder, falls noch nicht bekannt, Bezeichnung der Erfindung: /
Application No. or, if not yet known, title of the invention: /
N° de la demande ou, s'il n'est pas encore connu, titre de l'invention :

16195355.9

In Sachen der oben bezeichneten europäischen Patentanmeldung nennt (nennen) der (die) Unterzeichnete(n)[1] / In respect of the above European patent application I (we), the undersigned[1] / En ce qui concerne la demande de brevet européen susmentionnée, le(s) soussigné(s)[1]

Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510 / US

als Erfinder[2]: / do hereby designate as inventor(s)[2]: / désigne(nt) en tant qu'inventeur(s)[2] :

Mark J. EVANS
Radnor, Pennsylvania, US

Weitere Erfinder sind auf einem gesonderten Blatt angegeben. / Additional inventors are indicated on a supplementary sheet. /
D'autres inventeurs sont mentionnés sur une feuille supplémentaire.

Der (Die) Anmelder hat (haben) das Recht auf das europäische Patent erlangt[3] / The applicant(s) has (have) acquired the right to the European patent[3] /
Le(s) demandeur(s) a (ont) acquis le droit au brevet européen[3]

gemäß Vertrag vom /
by an agreement dated /
en vertu du contrat passé le

✓ als Arbeitgeber /
as employer(s) /
en qualité d'employeur(s)

durch Erbfolge /
as successor(s) in title /
par succession

Ort / Place / Lieu

Oxford, GB

Datum / Date

17 November 2022

Unterschrift(en) des (der) Anmelder(s) oder Vertreter(s): /
Signature(s) of applicant(s) or representative(s): /
Signature(s) du (des) demandeur(s) ou du (des) mandataire(s) :

Dr Pamela Tuxworth, Partner, Patent Attorney
J A Kemp LLP

Name des (der) Unterzeichneten bitte in Druckschrift wiederholen. Bei juristischen Personen bitte die Stellung des (der) Unterzeichneten innerhalb der Gesellschaft in Druckschrift angeben. / Please print name(s) under signature(s). In the case of legal persons, the position of the signatory within the company should also be printed. / Le ou les noms des signataires doivent être indiqués en caractères d'imprimerie. S'il s'agit d'une personne morale, la position occupée au sein de celle-ci par le ou les signataires doit également être indiquée en caractères d'imprimerie.

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Fußnoten zur Vorderseite

1 Name(n) des (der) Unterzeichneten nach Maßgabe der Regel 41 (2) c) und d) EPÜ:

Bei natürlichen Personen ist der Familienname vor den Vornamen anzugeben.
Bei juristischen Personen und Gesellschaften, die juristischen Personen gemäß dem für sie maßgebenden Recht gleichgestellt sind, ist die amtliche Bezeichnung anzugeben.

2 Name(n), Vorname(n), Wohnsitzstaat und Wohnort des Erfinders (der Erfinder) gemäß Regel 19 (1) EPÜ.
Der Wohnort entspricht der Stadt oder Gemeinde in der der Erfinder seinen Wohnsitz hat und schließt die Postleitzahl ein (sofern vorhanden).

3 Ist der Anmelder nicht oder nicht allein der Erfinder, so hat die Erfindernennung eine Erklärung darüber zu enthalten, wie der Anmelder das Recht auf das europäische Patent erlangt hat (Artikel 81, Regel 19 (1) EPÜ).

Bei rechtsgeschäftlicher Übertragung genügt die Angabe „gemäß Vertrag vom ...".

Bei Arbeitnehmererfindungen genügt der Hinweis, dass der oder die Erfinder Arbeitnehmer des Anmelders/der Anmelder ist bzw. sind.

Bei Erbfolge genügt die Angabe, dass der oder die Anmelder Erbe(n) des Erfinders/der Erfinder ist bzw. sind.

Footnotes to text overleaf

1 Name(s) of the undersigned in accordance with Rule 41(2)(c) and (d) EPC:

Names of natural persons shall be indicated by the person's family name, followed by his given names.
Names of legal persons, and of bodies equivalent to legal persons under the relevant law, shall be indicated by their official designations.

2 Family name(s), given name(s) and country and place(s) of residence of the inventor(s) in accordance with Rule 19(1) EPC.
The place of residence is the city or other municipality where the inventor permanently resides, and includes the postal code (where available).

3 If the applicant is not the inventor or is not the sole inventor, the designation shall contain a statement indicating the origin of the right to the European patent (Article 81, Rule 19(1) EPC).

In the case of assignment the words "by agreement dated ..." suffice.

In the case of inventions by employees a mention that the inventor(s) is/are employee(s) of the applicant(s) is sufficient.

In the case of succession a mention that the applicant(s) is/are heir(s) of the inventor(s) is sufficient.

Renvois concernant le texte figurant au recto

1 Nom(s) du (des) soussigné(s), conformément à la règle 41(2)c) et d) CBE :

Les personnnes physiques doivent être désignées par leurs noms suivis de leurs prénoms.
Les personnes morales et les sociétés assimilées aux personnes morales en vertu du droit dont elles relèvent doivent figurer sous leur désignation officielle.

2 Nom(s), prénom(s) de l'(des) inventeur(s) ainsi que le pays et le lieu de son (leur) domicile, conformément à la règle 19(1) CBE.
Le lieu de domicile correspond à la ville ou autre entité administrative où l'inventeur réside de façon permanente et inclut le code postal (lorsqu'il est disponible).

3 Si le demandeur n'est pas l'inventeur, ou l'unique inventeur, la désignation de l'inventeur doit comporter une déclaration indiquant l'origine de l'acquisition du droit au brevet européen (article 81 et règle 19(1) CBE).

En cas de transfert contractuel, il suffit de mentionner « en vertu du contrat passé le ... ».

Pour les inventions de salariés, il suffit d'indiquer que le ou les inventeurs sont des employés du ou des demandeurs.

En cas de transfert successoral, il suffit d'indiquer que le ou les demandeurs sont les héritiers du ou des inventeurs.

EPA/EPO/OEB 1002 02.21

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of the following subsequently filed document(s):

Submission number	11499199
Application number	EP16195355.9
Date of receipt	17 November 2022
Receiving Office	European Patent Office, The Hague
Your reference	N411599EP-B
Applicant	All applicants as on file
Documents submitted	package-data.xml epf1038.pdf (1 p.) INVT-1.pdf\Additional Inventor N411599EP-B PXT.pdf (1 p.) ep-sfd-request.xml F1002-1.pdf\Designation of Inventor N411599EP-B.pdf (2 p.)
Submitted by	CN=Elizabeth Davenport 55598
Method of submission	Online
Date and time receipt generated	17 November 2022, 15:42 (CET)
Message Digest	06:2E:85:F9:6F:13:B5:3D:03:F1:54:89:C8:C5:61:1C:F6:56:9F:8F

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J A Kemp LLP
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ROYAUME UNI

Formalities officer
Christensen, Jette

Date
24.11.2022

Reference	Application No./Patent No. 16195355.9 - 1112 / 3167888
Applicant/Proprietor Alexion Pharmaceuticals, Inc.	

Rectification of the designation of the inventor under Rule 21 EPC

The designation of the inventor has been rectified as requested. Your attention is drawn to the Notice from the European Patent Office dated 22 February 2021 concerning the amendment of Rules 19 and 143 EPC (OJ 2021, A12).

For the Legal Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

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