administration to use the remainder) or (ii) use of at least two vials for three patients. The same or similar practice conditions would result for a 350 mg dose regimen.

Yet skilled artisans would have been well aware that such approaches ran contrary to widely-accepted, best practices for safe injection in the U.S., *i.e.*, that "single-dose vials are intended for a single procedure or injection and should always be discarded at the end of the procedure/injection. They should never be stored for later use or subsequent procedures, even if for the same patient." (see Pugliese *et al.* (2010) *Am. J. Infect. Control* 38(10):789-798 and Figure 1 referencing Guidelines promulgated by the Centers for Disease Control, U.S. Department of Health and Human Services (Exhibit A). *See also* Dolan *et al.* (2010) *Am. J. Infect. Control* 38:167-172 (the authors advocating strict adherence to established best practices including "discard[ing] single use vials after use [and n]ever use them again for another patient") (Exhibit B)). These best practices were and continue to be an easily-implemented way to reduce the potential risk for inadvertent nosocomial transmission of sexually-transmitted diseases (*e.g.*, HIV), *e.g.*, by using auto-disposable syringes and taking "the vaccine or medicine from a single-dose vial." (see Gisselquist *et al.* (2003) *International J STD & AIDS* 14:717, 719 (Exhibit C)).

Thus, the teachings of '245 patent pointed to by the Examiner would appear to have led a skilled artisan away from the instantly-claimed dosage forms, since the '245 patent suggests that it would not have been convenient to administer lower dosage amounts of eculizumab in a 300 mg format. Accordingly, convenience simply cannot be a reason, let alone the reason, for a skilled artisan to have arrived at the claimed pharmaceutical compositions.

In sum, the Examiner has failed to show why a person of ordinary skill in the art would have thought to combine the cited references to arrive at the particularly claimed methods, which encompass a distinct subgenus of pharmaceutical compositions comprising a single unit dosage form of an anti-C5 antibody that inhibits cleavage of complement component C5 into fragments C5a and C5b, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody, wherein the single unit dosage form comprises a 10mg/ml solution of the anti-C5 antibody, and wherein the single unit dosage form has a volume of 30ml. Accordingly, claims 98, 100-106, 110-113, and 115 are patentable over the cited art.

VIII. APPENDIX OF CLAIMS

A copy of the claims involved in the present appeal is set forth in Appendix A.

IX. CONCLUSION

In view of the above arguments, Appellant urges the Examiner and the Board to reconsider and withdraw the current rejections and to pass the claims to allowance.

Appellant believes that the pending application is in condition for allowance. If additional fees are due, please charge our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN from with the undersigned is authorized to draw.

Dated: June 24, 2014

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON, MULLINS, RILEY & SCARBOROUGH, LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 202-4630
*617) 742-4214 (Fax)
Attorney/Agent For Appellants

APPENDIX A: CLAIMS

91. **(Previously Presented)** A method for treating a patient that:
(i) is afflicted with paroxysmal nocturnal hemoglobinuria (PNH) and
(ii) has been vaccinated against *Neisseria meningitides,* the method comprising administering to the patient an effective amount of a pharmaceutical composition comprising a single unit dosage form of an anti-C5 antibody that inhibits cleavage of complement component C5 into fragments C5a and C5b,
wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody,
wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody,
wherein the single unit dosage form has a volume of 30 mL,
and wherein the anti-C5 antibody is eculizumab.

98. **(Previously Presented)** The method according to claim 91, wherein the anti-C5 antibody is administered to the patient for at least 6 months.

100. **(Previously Presented)** The method according to claim 91, wherein the patient has a hemoglobin level less than (i) 14 g/dL if a man or (ii) 12 g/dL if a woman.

101. **(Previously Presented)** The method according to claim 91, wherein the patient has a hemoglobin level less than (i) 13 g/dL if a man or (ii) 11 g/dL if a woman.

102. **(Previously Presented)** The method according to claim 91, wherein the patient has a hemoglobin level less than (i) 12 g/dL if a man or (ii) 10 g/dL if a woman.

103. **(Previously Presented)** The method according to claim 91, wherein the patient suffers from hemolysis due to a C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.

104. **(Previously Presented)** The method according to claim 91, wherein the patient is anemic and the anemia results at least in part from extravascular hemolysis, and wherein the patient remains anemic following treatment with the anti-C5 antibody.

105. **(Previously Presented)** The method according to claim 91, wherein the patient is at least 18 years of age and has: (i) a PNH type III erythrocyte population of ≥ 10% and (ii) received at least four transfusions during the 12 months immediately preceding the first treatment with the anti-C5 antibody.

106. **(Previously Presented)** The method according to claim 91, wherein the patient has aplastic anemia or myelodysplastic syndrome.

110. **(Previously Presented)** The method according to claim 91, wherein the single unit dosage form is a preservative free formulation.

111. **(Previously Presented)** The method according to claim 91, wherein the pharmaceutical composition is administered to the patient under the following treatment schedule: (i) 600 mg of the antibody every 7 ± 2 days for the first 4 weeks, (ii) 900 mg of the antibody for the fifth dose 7 ± 2 days after (i), and (iii) 900 mg of the antibody every 14 ± 2 days thereafter, and wherein the administration occurs via 25 to 45 minute intravenous infusion.

112. **(Previously Presented)** A method for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), the method comprising:

vaccinating the patient against *Neisseria meningitides;* and

administering to the patient an effective amount of a pharmaceutical composition comprising a single unit dosage form of an anti-C5 antibody that inhibits cleavage of complement component C5 into fragments C5a and C5b, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody, wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody, wherein the single unit dosage form has a volume of 30 mL, and wherein the anti-C5 antibody is eculizumab.

113. **(Previously Presented)** A method for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), the method comprising administering to the patient an effective amount of a pharmaceutical composition comprising a single

unit dosage form of an anti-C5 antibody that inhibits cleavage of complement component C5 into fragments C5a and C5b, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody, wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody, wherein the single unit dosage form has a volume of 30 mL, and wherein the anti-C5 antibody is eculizumab.

115. **(Previously Presented)** A method for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), the method comprising administering to the patient a pharmaceutical composition comprising a single unit dosage form of an anti-C5 antibody under the following treatment schedule: (i) 600 mg of the antibody every 7 ± 2 days for the first 4 weeks, (ii) 900 mg of the antibody for the fifth dose 7 ± 2 days after (i), and (iii) 900 mg of the antibody every 14 ± 2 days thereafter, wherein the administration is to occur via 25 to 45 minute intravenous infusion, wherein the single unit dosage form comprises 300 mg of the anti-C5 antibody, wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody, wherein the single unit dosage form has a volume of 30 mL, wherein the antibody inhibits cleavage of complement component C5 into fragments C5a and C5b, and wherein the anti-C5 antibody is eculizumab.

APPENDIX B: EVIDENCE

Exhibit A is a copy of Pugliese *et al.* ((2010) *Am. J. Infect. Control* 38(10):789-798).

Exhibit B is a copy of Dolan *et al.* ((2010) *Am. J. Infect. Control* 38:167-172).

Exhibit C is a copy of Gisselquist *et al.* ((2003) *International J STD & AIDS* 14:717-722).

APPENDIX C: RELATED PROCEEDINGS

None.

Samsung Bioepis Exhibit No. 1002
Page 9597

Electronic Acknowledgement Receipt

EFS ID:	19390308
Application Number:	13426973
International Application Number:	
Confirmation Number:	8851
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard Bell
Customer Number:	959
Filer:	Jill R. Gorny Sloper
Filer Authorized By:	
Attorney Docket Number:	AXJ-114BUSCN
Receipt Date:	24-JUN-2014
Filing Date:	22-MAR-2012
Time Stamp:	17:08:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Appeal Brief Filed	ExhibitA.pdf	1143675 0b3e1c5601c987dd73c7a5c580c6e91de0ee5c1d	no	10

Warnings:	
Information:	

Samsung Bioepis Exhibit No. 1002
Page 9598

2	Appeal Brief Filed	ExhibitB.pdf	821591 5a8ebd9f0348eb96ce47e1f76869a53add993e96	no	6
Warnings:					
Information:					
3	Appeal Brief Filed	ExhibitC.pdf	636987 31418b0a537c1cdf79e9af37eaf1c9f8e06827da	no	6
Warnings:					
Information:					
4	Appeal Brief Filed	AppealBrief.pdf	63236 11e746a1a02bb8bb4983749423c4ee4b2a4318d3	no	14
Warnings:					
Information:					
		Total Files Size (in bytes):	2665489		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

Samsung Bioepis Exhibit No. 1002
Page 9599

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/426,973	03/22/2012	Leonard Bell	AXJ-114BUSCN	8851

959 7590 08/13/2014

NELSON MULLINS RILEY & SCARBOROUGH LLP
FLOOR 30, SUITE 3000
ONE POST OFFICE SQUARE
BOSTON, MA 02109

EXAMINER	
ROGERS, JAMES L	
ART UNIT	**PAPER NUMBER**
1644	

NOTIFICATION DATE	DELIVERY MODE
08/13/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
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BEFORE THE PATENT TRIAL AND APPEAL BOARD

Application Number: 13/426,973
Filing Date: March 22, 2012
Appellant(s): BELL ET AL.

Jill Gorny Sloper
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed June 24, 2014.

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Every ground of rejection set forth in the Office action dated January 27, 2014 from which the appeal is taken is being maintained by the examiner except for the grounds of rejection (if any) listed under the subheading "WITHDRAWN REJECTIONS." New grounds of rejection (if any) are provided under the subheading "NEW GROUNDS OF REJECTION."

(1) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

I. Claims 91, 98, 100-106, and 112-113 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen et al. (N Engl J Med 2004; 350: 552-9) and Hill et al. (Haematologica 2005), in view of Evans et al. (US 6,355,245 B1) and Wang (US 2005/0271660), as is evidenced by the current specification at p. 16, lines 20-25.

II. Claims 91, 98, 100-106, 110 and 112-113 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen et al. (N Engl J Med 2004; 350: 552-9) and Hill et al. (Haematologica 2005), in view of Evans et al. (US 6,355,245 B1) and Wang (US 2005/0271660), as applied to claims 91,98, 100-106, and 112-113 above, in further view of Garcia et al. (Biologicals (2002) 30, 143-151).

III. Claims 91, 98, 100-106, 111-113 and 115 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen et al. (N Engl J Med 2004; 350: 552-9) and Hill et al. (Haematologica 2005), in view of Evans et al. (US 6,355,245 B1) and Wang (US 2005/0271660), as applied to claims 91,98, 100-106, and 112-113 above, in further view of Rosse et al. (Hematology Am Soc Hematol Edc. Program, 2004, 48-62).

(2) Response to Argument

Appellant acknowledged that claims 91, 98, 100-106, 110-113, and 115, have been rejected under 35 U.S.C. 103(a), as outlined above. Appellant argues the rejection as follows:

Appellant asserts that none of the cited references teach or suggest a method, which includes a pharmaceutical composition having the particularly claimed limitations, i.e., wherein the composition comprises a single unit dosage form of 300 mg of the anti-C5 antibody, comprises a 10 mg/mL solution of the anti-C5 antibody, and that the single unit dosage form has a volume of 30mL. Appellant further asserts that one of ordinary skill in the art would not have been motivated to have selected the particularly claimed single unit dosage form (i.e., in which volume, concentration, and quantity are defined) out of the vast genus of potential dosage forms for eculizumab - e.g., which vary widely in volume, concentration, and quantity. Appellant asserts that the Court of Appeals for the Federal Circuit has been quite clear that, in such a case, a finding that this species is obvious cannot be predicated merely on the existence of myriad hypothetical possibilities (see, e.g., In re Baird, 16 F.3d 380 (Fed. Cir. 1994).

This argument was not considered persuasive because while the examiner acknowledges that neither of the primary references Hillmen (2004) or Hill (2005) recite the specific dose claimed by appellant (i.e., a single unit dosage form comprising 300 mg of the anti-C5 antibody, wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody in a volume of 30 mL), the '245 patent teaches that low doses of eculizumab which is the anti-C5 antibody are therapeutically effective (see p. 5

of the prior OA). The primary art reference Hill (2005) also teaches treating the same patient population afflicted with the same disease which is PNH by administering the same anti-C5 antibody, albeit at a higher dose of 600 mg (see p.3 of the prior OA). Those of skill in the art would look to the '245 patent for guidance in arriving at lower dosage amounts such as a single unit 300 mg or 10 mg/ml solution wherein the single unit dosage form has a volume of 30 mL as currently recited by appellant because the '245 teaches that lower dosages are also therapeutically effective.

The 1994 Federal Circuit case (i.e., Baird) cited by appellant does not concern drug doses but rather deals with whether a generic chemical formula estimated to encompass millions of compounds rendered obvious a claim to three species, particularly when the disclosure indicated a preference leading away from the claimed compounds. The issue in the current case, in contrast, concerns discovering optimal or workable drug doses in light of teachings of Hill with respect to treating the exact same patient population and the same disease with the same antibody. Where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges involves only routine skill in the art. In re Aller, 220 F2d 454, 456, 105 USPQ 233; 235 (CCPA 1955). See MPEP §§2144.05 part IIA. Given the teachings of Hill with respect to treating the exact same patient population and the same disease with the same antibody, arriving at appellants' dosage would have amounted to no more than routine skill in the art in light of the '245 patent teachings that eculizumab is also effective at lower concentrations than the 600 mg dose taught by Hill.

Appellants further submit that optimizing the dosage form is neither routine, obvious, nor a mere matter of convenience and that at a very minimum, to arrive at the claimed solutions, the inventors had to know the solubility properties of the claimed antibody, eculizumab.

This argument was not considered persuasive because Wang (US 2005/0271660) teaches that eculizumab is highly soluble, including at up to 30 mg per ml (paragraph 0171, Figure 10).

Appellant further asserts that for a pharmaceutical solution to be commercially viable, the inventors had to know the shelf-life stability of eculizumab in solution. If its stability was too low, a pharmaceutical solution would not have been an option. None of that knowledge is disclosed or suggested by the cited references. Appellant further asserts that weekly or biweekly injections of 154, 210, or 350 mg would require the use of multiple 300 mg vials of the instantly claimed formulation or multiple uses of the same vial. That is, doctors or nurses wishing to administer to a patient approximately 154 mg of an anti-C5 antibody formulated at 300 mg/vial would likely have had to either use the same vial: (a) more than once for the same patient or (b) for each of two patients or, otherwise, waste half of the vial's contents. To avoid the same product waste issue, the need to administer approximately 210 mg of an antibody to a patient would have necessitated the: (i) storage of a vial for potentially weeks (using only 210 mg of the 300rag vial for the first does and waiting for the second administration to use the remainder) or (ii) use of at least two vials for three patients. The same or similar practice conditions would result for a 350 mg dose regimen. Yet skilled artisans would have

been well aware that such approaches ran contrary to widely-accepted, best practices for safe injection in the U.S., i.e., that "single-dose vials are intended for a single procedure or injection and should always be discarded at the end of the procedure/injection. They should never be stored for later use or subsequent procedures, even if for the same patient." (see Pugliese et al. (2010) Am. J. Infect. Control 38(10):789-798 and Figure 1 referencing Guidelines promulgated by the Centers for Disease Control, U.S. Department of Health and Human Services (Exhibit A). See also Dolan et al. (2010) Am. J. Infect. Control 38:167-172 (the authors advocating strict adherence to established best practices including "discard[ing] single use vials after use [and n]ever use them again for another patient") (Exhibit B)). These best practices were and continue to be an easily-implemented way to reduce the potential risk for inadvertent nosocomial transmission of sexually-transmitted diseases (e.g., HIV), e.g., by using auto-disposable syringes and taking "the vaccine or medicine from a single-dose vial." (see Gisselquist et al. (2003) International J STD & AIDS 14:717,719 (Exhibit C)). Thus, the teachings of '245 patent pointed to by the Examiner would appear to have led a skilled artisan away from the instantly-claimed dosage forms, since the '245 patent suggests that it would not have been convenient to administer lower dosage amounts of eculizumab in a 300 mg format. Accordingly, convenience simply cannot be a reason, let alone the reason, for a skilled artisan to have arrived at the claimed pharmaceutical compositions.

This argument was also not considered persuasive because a known or obvious composition is not patentable simply because it has been described as somewhat

inferior to some other product for the same use. See In re Gurley 31 USPQ2d 1130, 1132 (Fed. Cir. 1994) and MPEP 2123. Given the teachings of the primary art references (i.e., Hillmen and Hill) as to treating the exact same patient population afflicted with the same disease which is PNH by administering the same anti-C5 antibody, albeit at a higher dose of 600 mg, those of skill in the art would look to the '245 patent in arriving at lower dosage amounts such as a single unit 300 mg or 10 mg/ML solution wherein the single unit dosage form has a volume of 30 mL. There would also have been a reasonable expectation of success in doing so because the '245 patent teaches that lower dosages are therapeutically effective.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

/James Rogers/

Conferees:

/DANIEL E. KOLKER/

Supervisory Patent Examiner, Art Unit 1644

/GARY NICKOL/

Supervisory Patent Examiner, Art Unit 1645

Requirement to pay appeal forwarding fee. In order to avoid dismissal of the instant appeal in any application or ex parte reexamination proceeding, 37 CFR 41.45 requires payment of an appeal forwarding fee within the time permitted by 37 CFR 41.45(a),

unless appellant had timely paid the fee for filing a brief required by 37 CFR 41.20(b) in effect on March 18, 2013.

Samsung Bioepis Exhibit No. 1002
Page 9608

Doc code: WFEE.APPEAL
Doc Description: Certification and Transmittal of Appeal Forwarding Fee

PTO/AIA/34 (06-14)

CERTIFICATION AND TRANSMITTAL OF APPEAL FORWARDING FEE	Docket Number (Optional) AXJ-114BUSCN

I hereby certify that this correspondence is being facsimile transmitted to the USPTO, EFS-Web transmitted to the USPTO, or deposited with the United States Postal Service with sufficient postage in an envelope addressed to "Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" [37 CFR 1.8(a)]
on August 27, 2014.
Signature /Jill Gorny Sloper, Esq./
Typed or printed name Jill Gorny Sloper, Esq.

In re Application of Leonard Bell	
Application Number 13/426,973	Filed March 22, 2012
For TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	
Art Unit 1644	Examiner J. L. Rogers

37 CFR 41.45 specifies that, in order to avoid dismissal of the appeal, appellant must pay the fee set in 37 CFR 41.20(b)(4) within two months of the later of the date of either the examiner's answer, or a decision refusing to grant a petition under 37 CFR 1.181 to designate a new ground of rejection in an examiner's answer.

The undersigned certifies that the appropriate fee accompanies this transmittal.

The fee for forwarding this appeal is (37 CFR 41.20(b)(4)) $ 2,000.00

☐ Appellant asserts small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by 50%, and the resulting fee is: $ _____

☐ Appellant certifies micro entity status. See 37 CFR 1.29. Therefore, the fee shown above is reduced by 75%, and the resulting fee is: $ _____
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 120080.

☑ Payment made via EFS-Web.

Extensions of time under 37 CFR 1.136(a) for patent applications are not applicable to the time period set forth in 37 CFR 41.45. See 37 CFR 1.136(b) for extensions of time to reply for patent applications and 37 CFR 1.550(c) for extensions of time to reply for *ex parte* reexamination proceedings.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ appellant ☑ attorney or agent of record Registration number 60,760 ☐ attorney or agent acting under 37 CFR 1.34 Registration number _____

Signature /Jill Gorny Sloper, Esq./
Typed or printed name Jill Gorny Sloper, Esq.
Telephone Number 617-573-4700
Date August 27, 2014

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☑ * Total of 1 forms are submitted.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Samsung Bioepis Exhibit No. 1002
Page 9609

Privacy Act Statement

The **Privacy Act of 1974** (**P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Samsung Bioepis Exhibit No. 1002
Page 9610

Electronic Patent Application Fee Transmittal

Application Number:	13426973
Filing Date:	22-Mar-2012
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard Bell
Filer:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Appeal Forwarding Fee	1413	1	2000	2000
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

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Page 9611

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**2000**

Samsung Bioepis Exhibit No. 1002
Page 9612

Electronic Acknowledgement Receipt	
EFS ID:	19980459
Application Number:	13426973
International Application Number:	
Confirmation Number:	8851
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard Bell
Customer Number:	959
Filer:	Jill R. Gorny Sloper
Filer Authorized By:	
Attorney Docket Number:	AXJ-114BUSCN
Receipt Date:	27-AUG-2014
Filing Date:	22-MAR-2012
Time Stamp:	17:36:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2000
RAM confirmation Number	4366
Deposit Account	120080
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

Samsung Bioepis Exhibit No. 1002
Page 9613

1	Transmittal Letter	Appeal_Fwd_Fee_Transmittal.pdf	223660 87938c69ce1ff0eae2f7cac02935bdf747a2a8d8	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	29935 0edf6f74b184bce369779f7397593b1a52669b6e	no	2
Warnings:					
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Page 9614

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/426,973	03/22/2012	Leonard Bell	AXJ-114BUSCN	8851

959 7590 10/28/2014

NELSON MULLINS RILEY & SCARBOROUGH LLP
FLOOR 30, SUITE 3000
ONE POST OFFICE SQUARE
BOSTON, MA 02109

EXAMINER	
ROGERS, JAMES L	
ART UNIT	**PAPER NUMBER**
1644	

NOTIFICATION DATE	DELIVERY MODE
10/28/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

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chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

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NELSON MULLINS RILEY & SCARBOROUGH LLP
FLOOR 30, SUITE 3000
ONE POST OFFICE SQUARE
BOSTON, MA 02109

Appeal No: 2015-000613
Application: 13/426,973
Appellant: Leonard Bell et al.

Patent Trial and Appeal Board Docketing Notice

Application 13/426,973 was received from the Technology Center at the Board on October 16, 2014 and has been assigned Appeal No: 2015-000613.

In all future communications regarding this appeal, please include both the application number and the appeal number.

The mailing address for the Board is:

PATENT TRIAL and APPEAL BOARD
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Telephone inquiries can be made by calling 571-272-9797 and referencing the appeal number listed above.

By order of the Patent Trial and Appeal Board.

MAT

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APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
13/426,973		1644	

OC000000078350603

Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 133485 on 07/16/2015

- **Correspondence Address**

The address of record for Customer Number 133485 is:

133485
Nelson Mullins Riley & Scarborough LLP/
Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/426,973	03/22/2012	Leonard Bell	AXJ-114BUSCN	8851

133485 7590 09/06/2016

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
09/06/2016	ELECTRONIC

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ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

Ex parte LEONARD BELL and RUSSELL P. ROTHER

Appeal 2015-000613
Application 13/426,973[1]
Technology Center 1600

Before DONALD E. ADAMS, JEFFREY N. FREDMAN, and RYAN H. FLAX, *Administrative Patent Judges*.

FLAX, *Administrative Patent Judge*.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134(a) involving claims directed to a method of treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) using eculizumab. Claims 91, 98, 100–106, 110–113, and 115 are on appeal as rejected under 35 U.S.C. § 103(a). We have jurisdiction under 35 U.S.C. § 6(b).

We affirm.

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[1] The Real Party in Interest is Alexion Pharmaceuticals, Inc. Br. 2.

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Page 9619

STATEMENT OF THE CASE

The appealed claims can be found in the Claims Appendix of the Appeal Brief. Claims 91, 112, 113, and 115 are independent claims. Claim 113 is representative and reads as follows:

> 113. A method for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), the method comprising administering to the patient an effective amount of a pharmaceutical composition comprising a single unit dosage form of an anti-C5 antibody that inhibits cleavage of complement component C5 into fragments C5a and C5b, wherein the single unit dosage form comprises 300 mg of the anti-CS antibody, wherein the single unit dosage form comprises a 10 mg/mL solution of the anti-C5 antibody, wherein the single unit dosage form has a volume of 30 mL, and wherein the anti-C5 antibody is eculizumab.

Br. 11–12 (Claims App'x).

The following rejections are on appeal:[2]

Claims 91, 98, 100–106, 112, and 113 rejected under 35 U.S.C. § 103(a) over Hillmen,[3] Hill,[4] Evans,[5] and Wang.[6] Final Action 2.

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[2] Each of the rejections set forth in the Final Action rely on the Examiner's determinations set forth in the Office Actions dated Aug. 20, 2012 and May 31, 2013. Final Action 2–4.

[3] Peter Hillmen et al., *Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria*, 350 NEW ENGLAND J. MED. 552–59 (2004) (hereinafter "Hillmen").

[4] Anita Hill et al., *Improvement in the Symptoms of Smooth Muscle Dystonia During Eculizumab Therapy in Paroxysmal Nocturnal Hemoglobinuria*, 90 HAEMATOLOGICA/THE HEMATOLOGY J. 111–13 (2005) (hereinafter "Hill").

[5] U.S. Patent No. US 6,355,245 B1 (issued to Mark J. Evans et al. Mar. 12, 2002) (hereinafter "Evans")

[6] U.S. Patent Application Pub. No. US 2005/0271660 A1 (published Dec. 8, 2005) (hereinafter "Wang").

Claims 91, 98, 100–106, 110, 112, and 113 rejected under 35 U.S.C. § 103(a) over Hillmen, Hill, Evans, Wang, and Garcia.[7] Final Action 4.

Claims 91, 98, 100–106, 111–113, and 115 rejected under 35 U.S.C. § 103(a) over Hillmen, Hill, Evans, Wang, and Rosse.[8]

We adopt the Examiner's findings of fact and reasoning regarding the scope and content of the prior art. For emphasis we highlight the following:

FINDINGS OF FACT

FF1. Hillmen disclosed, "patients with PNH received infusions of eculizumab (600 mg) every week for four weeks, followed one week later by a 900-mg dose and then by 900 mg every other week through week 12." Hillmen 552 and 554; *see also* Final Action 2–3, May 31, 2013 Office Action 3–7, and Ans. 3–7 (discussing Hillmen).

FF2. Hillmen disclosed, "Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components. It binds specifically to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b, thereby preventing the release of the inflammatory mediator C5a and the formation of the cytolyticpore C5b-C9." Hillmen 553; *see also* Final Action 2–3, May 31, 2013 Office Action 3–7, and Ans. 3–7 (discussing Hillmen).

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[7] Mildred Garcia et al., *Effect of Preservatives on IgG Aggregation, Complement-activating Effect and Hypotensive Activity of Horse Polyvalent Antivenom Used in Snakebite Envenomation*, 30 BIOLOGICALS 143–51 (2002) (hereinafter "Garcia").

[8] Wendell F. Rosse et al., *Immune-Mediated Hemolytic Anemia*, HEMATOLOGY, AM. SOC. HEMATOL. 48–62 (2004) (hereinafter "Rosse").

FF3. Hillmen disclosed "[a]ll patients were vaccinated against *N. meningitidis* (Mengivac (A+C), Aventis Pasteur) before treatment." Hillmen 554; *see also* Final Action 2–3, May 31, 2013 Office Action 3–7, and Ans. 3–7 (discussing Hillmen).

FF4. Hillmen disclosed "[t]here was a rapid improvement in the quality of life during eculizumab therapy." Hillmen 558, *see also* 557; *see also* Final Action 2–3, May 31, 2013 Office Action 3–7, and Ans. 3–7 (discussing Hillmen).

FF5. Hill corroborated the findings of Hillmen (*see supra*) and that eculizumab administered at a maintenance dose of 900 mg intravenously every 14 (or 12) days to treat PNH patients was safe, well tolerated, and effective. *See generally* Hill; *see also* Final Action 2–3, May 31, 2013 Office Action 4–7, and Ans. 3–7 (discussing Hill).

FF6. Wang disclosed "eculizumab in Formulation 1 (FIG. 10) at 30 mg/ml can be effectively and efficiently nebulized" for pulmonary drug delivery. Wang ¶ 171; *see also* May 31, 2013 Office Action 5, and Ans. 5 (discussing Wang).

FF7. Evans disclosed treating patients with "154, 210, or 350 mg of [eculizumab] antibody for a 70kg adult human." *See* Evans col. 16, ll. 10–30; *see also* Final Action 3, May 31, 2013 Office Action 5, and Ans. 4–6 (discussing Evans).

FF8. Rosse disclosed:

> Eculizumab is a humanized chimeric antibody against C5 with a completely nonfunctional Fe domain. Eculizumab has a high affinity for C5 and thus when bound remains so until the complex is removed from the circulation. Recently a pilot study of eculizumab in 11 patients with transfusion-dependent PNH was

> reported. The antibody was given at a dose of 600 mg every week for 4 weeks and then at a dose of 900 mg every other week. The drug is given by intravenous infusion over 30 minutes and was extremely well tolerated. . . . There was a significant improvement in the quality of life when comparing before the start of eculizumab and at week 12.

Rosse 53; *see also* Final Action 2–3, May 31, 2013 Office Action 7–8, and Aug. 20, 2012 Office Action 3–10 (discussing Rosse).

DISCUSSION

We address the three obviousness rejections together because the same determinative facts and arguments apply to each.

Appellants contend the Examiner's rejection fails to "show some *reason* why a person of ordinary skill in the art would have thought to combine *particular* available elements of knowledge, as evidenced by the prior art, to reach the claimed invention." Br. 6 (emphasis original). Further, Appellants contend "[n]one of the cited references teach or suggest a method, which includes a pharmaceutical composition having the particularly claimed limitations," i.e., a single unit dosage of 300 mg eculizumab in 30 mL of a 10 mg/mL solution. Br. 6–7. Appellants argue:

> optimizing the dosage form . . . is neither routine, obvious, nor a mere matter of convenience. At a very minimum, to arrive at the claimed solutions, the inventors had to know the solubility properties of the claimed antibody, eculizumab. Moreover, for a pharmaceutical solution to be commercially viable, the inventors had to know the shelf-life stability of eculizumab in solution. If its stability was too low, a pharmaceutical solution would not have been an option. None of that knowledge is disclosed or suggested by the cited references.

Br. 7.

The Examiner has set forth a prima facie case for obviousness, as the cited prior art disclose all but the specific single unit dosage of the claims. *See, e.g.*, FF1–FF8. A 300 mg single unit dosage would be obvious over effective working examples requiring therapeutic dosages that are simple multiples thereof, i.e., the effective 600 mg and 900 mg dosages of Hillmen, Hill, and Rosse (a 300 mg dose would be a convenience). *Id.* Moreover, the Examiner has determined that the point of distinction identified by Appellants, that is, the single unit dosage of 300 mg of eculizumab in a 30 mL formulation, i.e., at a concentration of 10 mg/mL, was obvious as mere optimization of the formulations disclosed by the combined prior art and achievable by routine experimentation. Ans. 4. A preponderance of the evidence of record supports the Examiner's determinations.

"[I]t is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456 (CCPA 1955); *see also In re Peterson*, 315 F.3d 1325 (Fed. Cir. 2003). "Only if the 'results of optimizing a variable' are 'unexpectedly good' can a patent be obtained for the claimed critical range." *In re Geisler*, 116 F.3d 1465, 1469 (Fed. Cir. 1997) (quoting *In re Antonie*, 559 F.2d 618, 620 (CCPA 1977)). "[D]iscovery of an optimum value of a result effective variable in a known process is ordinarily within the skill of the art." *In re Boesch*, 617 F.2d 272, 276 (CCPA 1980). Appellants' claims and arguments fail under these tests.

Contrary to Appellants' arguments, the claims do not recite that their pharmaceutical composition is commercially viable or has a specific shelf-life or has a specific stability. Nor does the Specification discuss these qualities or suggest that they were even considered by Appellants in relation

to the single unit dosage of the claims. Similarly, the Appellants-argued points on mitigation of waste, and storage, waiting, and drug vial multiplication do not address claim limitations and are not discussed in the Specification. *See* Br. 7–8. In fact, the Specification does not indicate a single advantage of the single unit dosage of the claims. Moreover, Appellants present no compelling evidence of secondary indicia of non-obviousness to rebut the Examiner's prima facie case of obviousness. Thus, there is no evidence that the optimization of the single unit dosage was anything but routine and it is well settled that arguments of counsel cannot take the place of factually supported objective evidence. *See*, *e.g.*, *In re Huang*, 100 F.3d 135, 139-40 (Fed. Cir. 1996); *In re De Blauwe*, 736 F.2d 699, 705 (Fed. Cir. 1984).

Where, as here, the experimentation needed to arrive at the subject matter claimed was "nothing more than routine" application of a well-known problem-solving strategy, we must conclude it is not invention. *Pfizer, Inc. v. Apotex, Inc.*, 480 F.3d 1348, 1368 (Fed. Cir. 2007).

For the above reasons, we find that the evidence of record supports the Examiner's determination that the claims would have been obvious over the cited prior art combination. The claims were not separately argued by Appellants and fall as a group. 37 C.F.R. § 41.37(c)(1)(iv).

SUMMARY

The rejection claims 91, 98, 100–106, 112, and 113 under 35 U.S.C. § 103(a) over Hillmen, Hill, Evans, and Wang is affirmed.

The rejection of claims 91, 98, 100–106, 110, 112, and 113 under 35 U.S.C. § 103(a) over Hillmen, Hill, Evans, Wang, and Garcia is affirmed.

The rejection of claims 91, 98, 100–106, 111–113, and 115 under 35 U.S.C. § 103(a) over Hillmen, Hill, Evans, Wang, and Rosse is affirmed.

TIME PERIOD FOR RESPONSE

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a).

AFFIRMED

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/426,973	03/22/2012	Leonard Bell	AXJ-114BUSCN	8851

133485 7590 11/15/2016

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
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ipqualityassuranceboston@nelsonmullins.com

Notice of Abandonment	Application No.	Applicant(s)
	13/426,973	BELL ET AL.
	Examiner	Art Unit
	JAMES ROGERS	1644

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☐ Applicant's failure to timely file a proper reply to the Office letter mailed on _____.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 to the final rejection.
   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) if this is utility or plant application, a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. Note that RCEs are not permitted in design applications.)
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☐ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $_____ is insufficient. A balance of $_____ is due.
   The issue fee required by 37 CFR 1.18 is $_____. The publication fee, if required by 37 CFR 1.18(d), is $_____.
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record or other party authorized under 37 CFR 1.33(b). See 37 CFR 1.138(b).

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34) upon the filing of a continuing application.

6. ☒ The decision by the Board of Patent Appeals and Interference rendered on *9/6/2016* and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☐ The reason(s) below:

/DANIEL E. KOLKER/
Supervisory Patent Examiner, Art Unit 1644

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Page 9629

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Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Leonard BELL
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	AXJ-114BUSCN2

SIGNATURE of Applicant or Patent Practitioner

Signature	/Jill Gorny Sloper, Esq./	Date (Optional)	May 6, 2016
Name	Jill Gorny Sloper, Esq.	Registration Number	60,760
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of 1 forms are submitted.

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Dated: May 6, 2016 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Doc Code: PA..
Document Description: Power of Attorney

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I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

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OR

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OR

Firm or Individual Name	Jane E. Remillard, Esq. NELSON MULLINS RILEY & SCARBOROUGH LLP				
Address	One Post Office Square				
City	Boston	State	MA	Zip	02109-2127
Country	US				
Telephone	(800) 237-2000	Email	IPBoston.Docketing@nelsonmullins.com		

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Alexion Pharmaceuticals, Inc.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	07/20/15
Name	Todd Spalding		
Title	Vice President, Head of Intellectual Property		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted.

Samsung Bioepis Exhibit No. 1002
Page 9631

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REQUEST FOR PARTICIPATION IN THE GLOBAL/IP5 PATENT PROSECUTION HIGHWAY (PPH) PILOT PROGRAM IN THE USPTO

Application No.:	Not Yet Assigned	First Named Inventor:	Leonard BELL
Filing Date:	Concurrently Herewith	Attorney Docket No.:	AXJ-114BUSCN2
Title of the Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT		

THIS REQUEST FOR PARTICIPATION IN THE PPH PILOT PROGRAM ALONG WITH THE REQUIRED DOCUMENTS MUST BE SUBMITTED VIA EFS-WEB. INFORMATION REGARDING EFS-WEB IS AVAILABLE AT HTTP://WWW.USPTO.GOV/PATENTS-APPLICATION-PROCESS/FILING-ONLINE/ABOUT-EFS-WEB.

APPLICANT HEREBY REQUESTS PARTICIPATION IN THE PATENT PROSECUTION HIGHWAY (PPH) PILOT PROGRAM AND PETITIONS TO MAKE THE ABOVE-IDENTIFIED APPLICATION SPECIAL UNDER THE PPH PILOT PROGRAM.

Office of earlier examination (OEE): Europe (European Patent Office)

OEE application number: 11001632.6

Both the OEE application and the above-identified U.S. application have the following earliest date (filing or priority date): March 15, 2006

Type of OEE work product relied upon: EPO Form 2906

Mailing date of OEE work product: July 22, 2015

Supporting Documents

One or more of the required documents below may be available electronically from the Dossier Access System or PATENTSCOPE (see https://www.jpo.go.jp/ppph-portal/filewrapper.htm). If the applicant requests the USPTO to attempt to obtain a document electronically and such attempt is unsuccessful, the applicant will be required to supply the document. Accordingly, to avoid dismissal of the initial PPH request and potential denial of participation in the PPH program, the applicant should verify that the document is actually available via the Dossier Access System or PATENTSCOPE before requesting retrieval. If the applicant is unable to verify availability, then the applicant should submit the document with the PPH request.

1. OEE Work Product and Translation

A copy of the OEE work product and translation if not already in English:

☐ Attached ☐ Previously submitted ☐ Not required because the decision to grant a patent was the first office action

☒ Applicant requests the USPTO to attempt to obtain the document(s) from the Dossier Access System or PATENTSCOPE

2. References Cited in OEE Work Product

A listing of references cited in the OEE work product and document copies (except U.S. patents and U.S. published patent applications):

☒ Attached ☐ Previously Submitted ☐ Not required because no references were cited in the OEE work product

☒ Applicant requests the USPTO to attempt to obtain the document(s) from the Dossier Access System or PATENTSCOPE

PTO/SB/20GLBL (04-15)
Approved for use through 04/30/2018. OMB 0651-0058
U.S. Patent and Trademark Office; U.S DEPARTMENT OF COMMERCE
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REQUEST FOR PARTICIPATION IN THE GLOBAL/IP5 PPH PILOT PROGRAM IN THE USPTO
(continued)

Application No.:	Not Yet Assigned	First Named Inventor:	Leonard BELL

3. Claims Correspondence Certification Statement

All of the claims in this application sufficiently correspond to the patentable/allowable claims in the OEE application.

4. Claims Correspondence Table

Claims in US Application	Patentable Claims in OEE Application	Explanation regarding the correspondence
1	1	Both claims are identical.

Signature	/Jill Gorny Sloper, Esq./	Date	May 6, 2016
Name (Print/Typed)	Jill Gorny Sloper, Esq.	Registration Number	60,760

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 6, 2016 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

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 6, 2016
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.: Not Yet Assigned

Confirmation No.: N/A

Filed: Concurrently Herewith

Art Unit: N/A

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

Examiner: Not Yet Assigned

INFORMATION DISCLOSURE STATEMENT (IDS)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In compliance with 37 C.F.R. § 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached PTO/SB/08.

For the Examiner's convenience in reviewing this continuation application, Applicant submits a consolidated PTO Form SB/08, listing all references cited during the prosecution of the parent application. The present application is a continuation of U.S. Application No. 13/426,973, filed March 22, 2012 (Atty. Docket No. AXJ-114BUSCN). In accordance with 37 C.F.R. §1.98(d), copies of the references previously cited by or submitted to the Office in the parent application are not enclosed, but will be provided upon request. In accordance with 37 C.F.R. § 1.98(a)(2)(i)-(iv), Applicant submits herewith copies of a non-patent literature references.

Samsung Bioepis Exhibit No. 1002
Page 9634

It is respectfully requested that the documents listed on the PTO/SB/08 be expressly considered by the Examiner during the prosecution of this application, and that the documents be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

Applicants call to the attention of the Examiner the following Applications and Office Actions cited therein:

Applications				
Examiner's Initials	*Serial No.*	*Filing Date*	*First Named Inventor*	*Docket No.*
	13/426,973	03-22-2012	Leonard Bell	AXJ-114BUSCN
	12/225,040	05-13-2009	Leonard Bell	AXJ-114BUS
	12/621,792	11-19-2009	Leonard Bell	AXJ-114DV
	10/771,552	02-03-2004	Leonard Bell	AXJ-114RCE
	11/050,543	02-03-2005	Leonard Bell	AXJ-114CPRCE
	11/595,118	11-08-2006	Leonard Bell	AXJ-114CP2
	12/902,617	10-12-2010	Leonard Bell	AXJ-114CPCN
	13/335,518	12-22-2011	Leonard Bell	AXJ-114CPCN2RCE

Office Actions (copies enclosed)			
Examiner's Initials	Serial No.	Date Mailed from USPTO	Examiner
	13/426,973	01-27-2014	J. Rogers
	13/426,973	05-31-2013	J. Rogers
	13/426,973	08-20-2012	J. Rogers
	12/225,040	09-22-2011	J. Rogers
	12/225,040	06-01-2011	J. Rogers
	12/225,040	04-11-2011	J. Rogers
	12/621,792	09-30-2010	P. Gambel
	10/771,552	12-22-2008	F. Vandervegt
	10/771,552	03-21-2008	F. Vandervegt
	10/771,552	08-10-2007	F. Vandervegt
	10/771,552	02-26-2007	F. Vandervegt
	10/771,552	09-26-2006	F. Vandervegt
	11/050,543	07-09-2010	P. Gambel
	11/050,543	04-02-2008	F. Vandervegt
	11/050,543	10-10-2007	F. Vandervegt
	11/050,543	06-11-2007	F. Vandervegt
	11/595,118	01-21-2011	P. Gambel
	11/595,118	10-05-2010	P. Gambel

	11/595,118	07-07-2010	P. Gambel
	11/595,118	04-03-2009	F. Vandervegt
	12/902,617	06-23-2011	P. Gambel
	12/902,617	04-04-2011	P. Gambel
	13/335,518	09-12-2014	P. Gambel
	13/335,518	02-06-2014	P. Gambel
	13/335,518	11-07-2013	P. Gambel

The filing of this Information Disclosure Statement is not to be interpreted as a representation that the cited documents are material, that an exhaustive search has been conducted, or that no other relevant information exists. Nor shall the citation of any documents herein be construed *per se* as a representation that such document is prior art. Moreover, Applicant understands the Examiner will make an independent evaluation of the cited documents.

This Information Disclosure Statement accompanies the new patent application submitted herewith. Accordingly, no fees are due with this submission (37 C.F.R § 1.97(b)(3)). However, the Director is hereby authorized to charge any fee deficiency which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2.

Dated: May 6, 2016

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY &
SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

PTO/SB/08a (03-15)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	Leonard BELL
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	AXJ-114BUSCN2

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	4686100		1987-08-11	Raffin et al.	
	2	5135916		1992-08-04	Sims et al.	
	3	5660825		1997-08-26	Sims et al.	
	4	6355245		2002-03-12	Evans et al.	
	5	7361339		2008-04-22	Bell	
	6	7482435		2009-01-27	Bowdish et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear

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	1	20030049683	A1	2003-03-13	Bowdish et al.	
	2	20050191298	A1	2005-09-01	Bell et al.	
	3	20050221382	A1	2005-10-06	Rother	
	4	20050169921	A1	2005-08-04	Bell et al.	
	5	20050271660	A1	2005-12-08	Wang	
	6	20070116710	A1	2007-05-24	Bell et al.	
	7	20090220508	A1	2009-09-03	Bell et al.	
	8	20100068202	A1	2010-03-18	Bell et al.	
	9	20110086040	A1	2011-04-14	Bell et al.	
	10	20120308559	A1	2012-12-06	Bell et al.	
	11	20120237515	A1	2012-09-20	Bell et al.	

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	0411306	EP	A1	1991-02-06	Goetze, Otto Prof. Dr. Med		
	2	2004/029207	WO	A2	2004-04-08	Xencor Inc		
	3	2005074607	WO	A2	2005-08-18	Alexion Pharmaceuticals, Inc		

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	Alexion Announces Presentation of Preliminary Results from Phase I Pilot Study of Eculizumab Treatment of Patients with Paroxysmal Nocturnal Hemoglobinuria"; [online]; Alexion Pharmaceuticals: News; [Retrieved on 2003-06-18]. Retrieved from the world wide web at alexionpharmaceuticals.com/news/sub press.cfm?prid=242&selectyear=2002.	
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	3	Alexion Pharmaceuticals: Complement Inhibitors; [online]; [Retrieved on 2003-06-17]; Retrieved from the world wide web at alexionpharm.com/techplat/complement.cfm.	
	4	Alexion Pharmaceuticals Signs Agreement with Lonza Biologics for Commercial Manufacturing; [online]; Alexion Pharmaceuitcals Inc.; [Retrieved on 2003-06-17]; Retrieved from the world wide web at noonanrusso.com/news/view newsitem.aspx?ItemID=394.	

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	5	Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoglobinuria, TRANSPLANT NEWS, 01-15-2004, XP002497241;Retrieved from the world wide web at findarticles. com/p/articles/mi mOYUG/is 1 14/ai 17208612 [retrieved on 09-25-2008]	
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	16	MORRIS J T ET AL: "Recurrence of neisserial meningococcemia due to deficiency of terminal complement component," Database accession no. NLM1411724, DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; October 1992 (1992-10)	
	17	Figueroa and Densen, "Infectious Diseases Associated with Complement Deficiencies," Clinical Microbiology Reviews, vol. 4(3): 359-395 (1991).	
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	24	Hill et al., "Improvement in the symptoms of smooth muscle dystonia during eculizumab therapy in paroxysmal nocturnal hemoglobinuria," Haematology, 90(12): Abstract #ECR40; (2005)	
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	27	Hill, et al., "Protection of erythrocytes from human complement-mediated lysis by membranetargeted recombinant soluble CD59: a new approach to PNH therapy," Blood 107(5):2131-2137 (2006).	
	28	Hillmen et al. "Effect of Eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal Hemoglobinuria," N Engl J Med, vol. 350(6):552-559 (2004)	
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	31	Hill, "Eculizumab, for the treatment of paroxysmal nocturnal hemglobinuria," Clin. Adv. In Hemal. & Onc., XP0015384263 (11):849-850 (2005) .	
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	47	MERCK MANUAL of Diagnosis and Therapy (The), Seventeenth Edition, Chapter 127 Anemias, 849-883 (1999).	
	48	Hillmen et al., "Eculizumab, a C5 Complement-Blocking Antibody, Controls Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Responses Maintained over prolonged Period of Therapy" Journal of The American Society of Hematology, Vol. 102(11), Abstract 1858 (2003).	

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	49	Hillmen et al., "Eculizumab, a C5 complement-blocking antibody, controls haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged period of therapy," British Journal of Haematology, Vol. 125 (Suppl. 1): 1-72, Abstract 5 (2004).	

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2016-05-06
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60760

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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	First Named Inventor	Leonard BELL
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

	1	Mortazavi et al., "The spectrum of PIG-A gene mutations in a plastic anemia/paroxysmal nocturnal hemoglobinuria (AAIPNH): a high incidence of multiple mutations and evidence of a mutational hot spot," Blood 101 2833-2841 (2003).	
	2	Moyo et al., "Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays," British J Haematology 126, 133-138 (2004).	
	3	Notice of Opposition for EP Patent No. 1720571, dated 3/18/2013.	
	4	Parker et al., Diagnosis and management of paroxysmal nocturnal hemoglobinuria, Blood, 106(12):3699-3709 (2005).	
	5	Paroxysmal nocturnal hemoglobinuria, included; PNH, included phosphatidylinositol glycan, class A, pseudogene 1, included; pigap1, included piga-related processed gene, included [online]. OM 1M-Online Medelian Inheritance in Man; John Hopkins University; [Retrieved on 2003-06-18]. Retrieved from the world wide web at ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=OMIM&dopt=detailed ...	
	6	Reiter et al., "Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease," Nature Medicine 8 (12):1383-1389 (2002).	
	7	Richards et al., "Evolution of GPI-Deficient Clones Predicts Clinical Course in Paroxysmal Nocturnal Haemoglobinuria," Blood, 104:Abstract 172 (2004).	
	8	Rodary et al., "Patient preference for either the EORTC QLQ-C30 or the FACIT Quality of Life (QOL) measures: a study performed in patient suffering from carcinoma of an unknown primary site (CUP)," European Journal of Cancer, Vol. 40, pp. 521-528 (2004).	
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	10	ROTHER et al., Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH), Molecular Immunology, Vol. 40(2-4), page 197; XP009163498, (2003).	
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	First Named Inventor	Leonard BELL
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	12	Stebler et al., High-dose recombinant human erythropoietin for treatment of anemia in myelodysplastic syndromes and paroxysmal nocturnal hemoglobinuria: a pilot study, Exp. Hematol. 18:1204-1208 (1990).	
	13	Strauss et al. "Alternative pathway of complement in sickle cell disease," Pediat. Res., vol. 11:285-289 (1977)	
	14	Stuart et al., "Sickle-cell disease," Lancet, vol. 364:1343-60 (2004)	
	15	Tang and Pikal, Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice, Pharmaceutical Research, Vol. 21 (2):191-200 (2004).	
	16	Thomas et al., Inhibition of complement activity by humanized anti-C5 antibody and single chain Fv, Mol.Imm. 33 (17-18):1389-1401, XP002262113 (1996).	
	17	Vakeva, et al., "Myocardial infarction and apoptosis after myocardial ischemia and reperfusion," Circulation 97:2259-2267 (1998).	

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Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Filer:	Jill R. Gorny Sloper/Carolyn Blake
Attorney Docket Number:	AXJ-114BUSCN2
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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
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Application Number:	15148839
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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.

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.

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

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.

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

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

Treatment Group	LDH Category	Change of FACIT from Baseline		
		<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.

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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Ref Type: Abstract

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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
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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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WHAT IS CLAIMED IS:

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution.

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TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

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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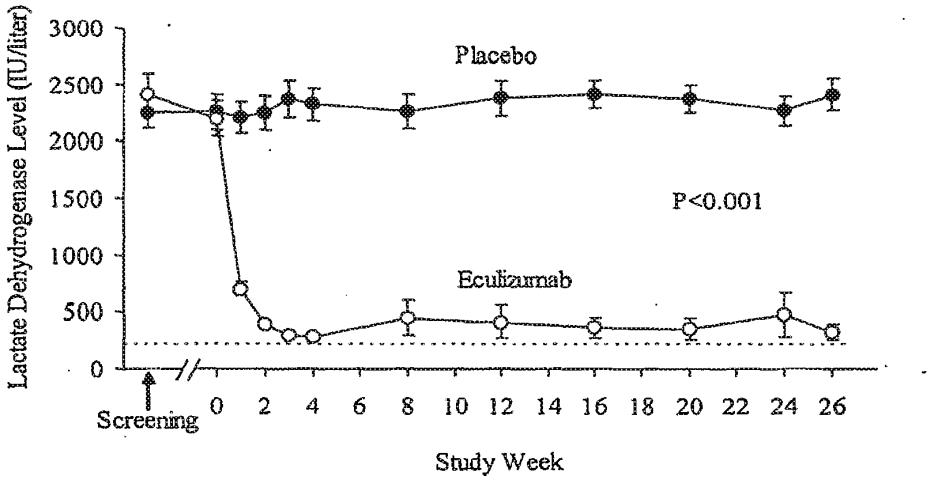

Lactate Dehydrogenase Level (IU/liter)
3000
2500
2000
1500
1000
500
0
Placebo
P<0.001
Eculizumab
Screening
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 1A

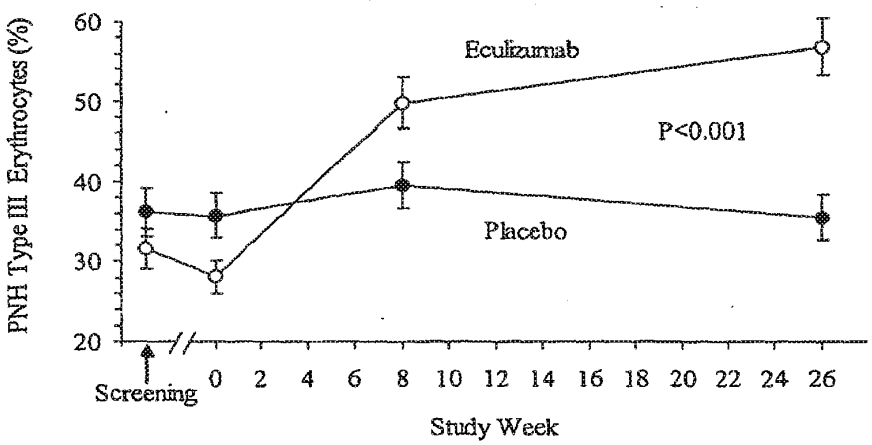


FIGURE 1B

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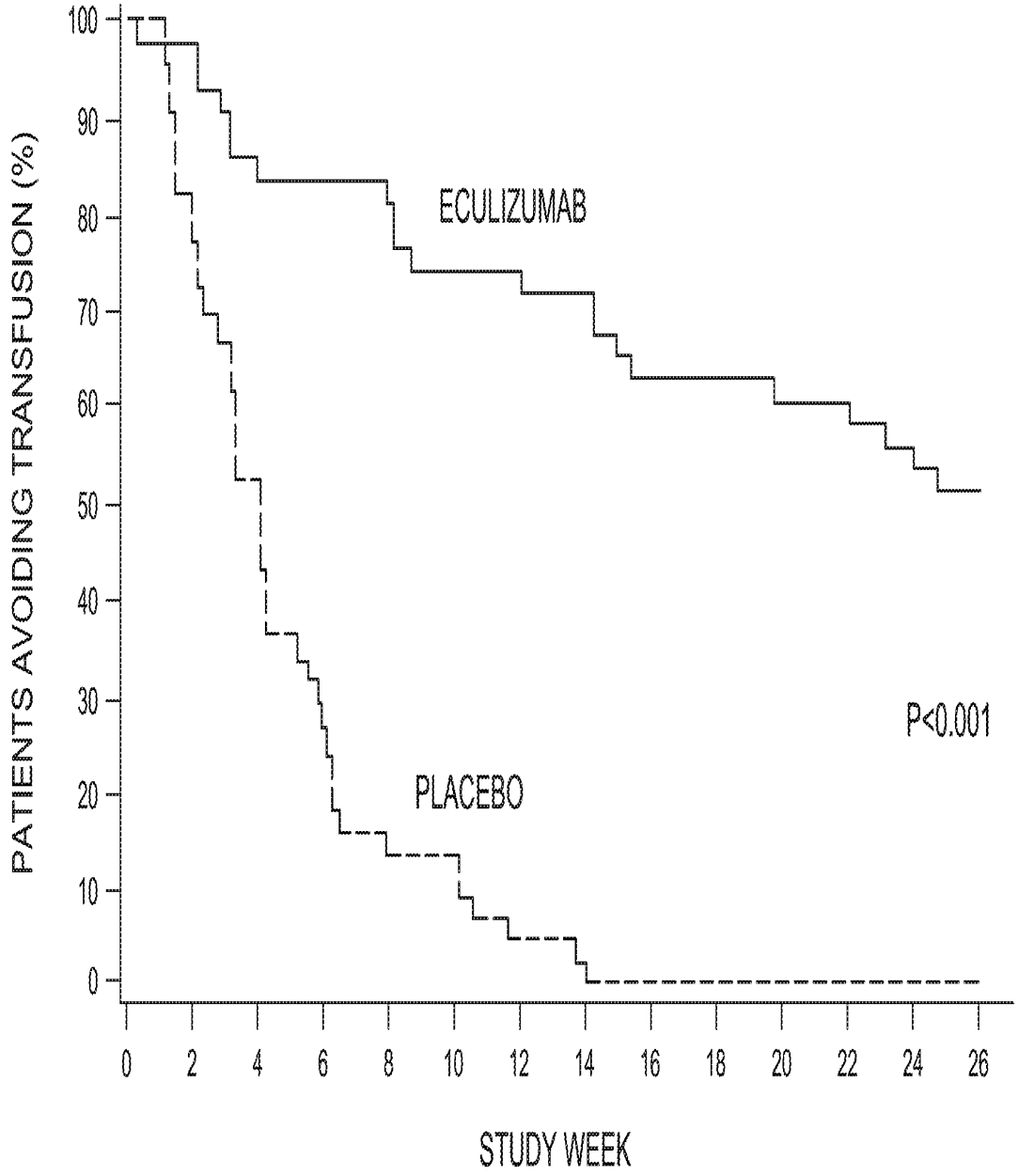

PATIENTS AVOIDING TRANSFUSION (%)
100
90
80
70
60
50
40
30
20
10
0
ECULIZUMAB
PLACEBO
P<0.001
0
2
4
6
8
10
12
14
16
18
20
22
24
26
STUDY WEEK

FIGURE 2

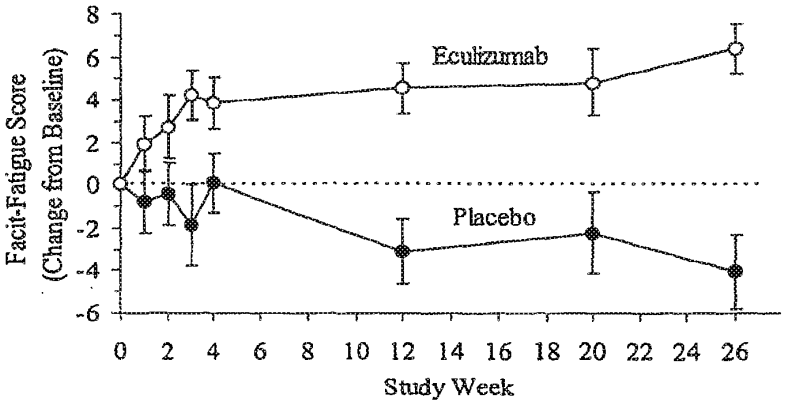


FIGURE 3

Samsung Bioepis Exhibit No. 1002
Page 9709

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 6, 2016
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.: Not Yet Assigned

Confirmation No.: N/A

Filed: Concurrently Herewith

Art Unit: N/A

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

Examiner: Not Yet Assigned

MS Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT PURSUANT TO 37 CFR 1.821

Dear Sir:

Submitted herewith in connection with the above-referenced patent application and in full compliance with 37 C.F.R. §§1.821-1.825 is a computer readable copy and paper copy of the Sequence Listing (filed together as a .txt file via the United States Patent Office's Electronic Filing System).

I hereby state that I have reviewed the paper copy of the Sequence Listing, as required by 37 CFR 1.821(c), and have reviewed the computer readable form of the Sequence Listing, as required by 37 CFR 1.821(e), and that the content of the paper and computer readable copies for the above-referenced patent application are the same as required by 37 CFR 1.821(f) (note that these documents are submitted as one electronic file). No new matter has been added to the Sequence Listing.

Samsung Bioepis Exhibit No. 1002
Page 9710

Dated: May 6, 2016

Respectfully submitted,
Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY &
SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Leonard		BELL	

Residence Information (Select One) • US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Woodbridge	CT	US

Mailing Address of Inventor:

Address 1	59 Tumblebrook Road
Address 2	

City	State/Province	Postal Code	Country
Woodbridge	CT	06525	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Russell	P.	ROTHER	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Oklahoma City	OK	US

Mailing Address of Inventor:

Address 1	10325 Forest Meadow Drive
Address 2	

City	State/Province	Postal Code	Country
Oklahoma City	OK	73151	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	133485		
Email Address	IPBoston.Docketing@nelsonmullins.com	Add Email	Remove Email

Application Information:

Title of the Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT		
Attorney Docket Number	AXJ-114BUSCN2	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	4	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	133485		

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	13/426973	2012-03-22
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13/426973	Continuation of	12/225040	2009-05-13
Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
12/225040	a 371 of international	PCT/US2007/006606	2007-03-15
Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2007/006606	Claims benefit of provisional	60/783070	2006-03-15

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 — Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Alexion Pharmaceuticals, Inc.		
Mailing Address Information For Applicant:			
Address 1	100 College St.		
Address 2			
City	New Haven	**State/Province**	CT
Country	US	Postal Code	06510
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Jill Gorny Sloper, Esq./			Date (YYYY-MM-DD)	2016-05-06
First Name	Jill Gorny	Last Name	Sloper	Registration Number	60760

Additional Signature may be generated within this form by selecting the Add button. Add

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN2
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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============================================================
Sequence Listing was accepted.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Saleem, Syed (ASRC)
Timestamp: [year=2016; month=5; day=12; hr=13; min=50; sec=42; ms=843; ]
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Page 9721

Validated By CRFValidator v 1.0.5

Application No: 15148839 Version No: 1.0

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Output Set:

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Page 9722

SEQUENCE LISTING

<110> BELL, LEONARD
ROTHER, RUSSELL P.

<120> TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS
BY AN INHIBITOR OF COMPLEMENT

<130> AXJ-114BUSCN2

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35 40 45

Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
50 55 60

Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65 70 75 80

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Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

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Trp Ile Gln Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
    50                  55                  60

Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65                  70                  75                  80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Tyr Phe Phe Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val Trp
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Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
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    130                 135                 140

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145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
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Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
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Val Pro Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp
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His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys
    210                 215                 220

Cys Val Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser
225                 230                 235                 240

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                245                 250                 255

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro
            260                 265                 270

Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
        275                 280                 285

Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val
    290                 295                 300

Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
305                 310                 315                 320

Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr
                325                 330                 335

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
            340                 345                 350

Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys
        355                 360                 365
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Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
    370                 375                 380

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
385                 390                 395                 400

Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser
                405                 410                 415

Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
            420                 425                 430

Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
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            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Gly Ala Ser
        35                  40                  45

Glu Asn Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

Ala Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn
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Page 9726

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            100                 105                 110

Val Leu Asn Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
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Lys Arg Thr
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            20                  25                  30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Gly Ala Ser
        35                  40                  45

Glu Asn Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

Ala Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn
            100                 105                 110

Val Leu Asn Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
        115                 120                 125

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp
    130                 135                 140
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Page 9727

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Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn
145                 150                 155                 160

Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu
                165                 170                 175

Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp
            180                 185                 190

Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr
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Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
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Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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Asp



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Samsung Bioepis Exhibit No. 1002
Page 9728

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Page 9729

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/148,839	05/06/2016	1677	1740	AXJ-114BUSCN2	1	1

CONFIRMATION NO. 3159

FILING RECEIPT

133485
Nelson Mullins Riley & Scarborough LLP/
Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

Date Mailed: 05/17/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Leonard BELL, Woodbridge, CT;
Russell P. ROTHER, Oklahoma City, OK;

Applicant(s)

Alexion Pharmaceuticals, Inc., New Haven, CT;

Power of Attorney: The patent practitioners associated with Customer Number 133485

Domestic Priority data as claimed by applicant

This application is a CON of 13/426,973 03/22/2012
which is a CON of 12/225,040 05/13/2009 ABN
which is a 371 of PCT/US2007/006606 03/15/2007
which claims benefit of 60/783,070 03/15/2006

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 05/16/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/148,839**

Projected Publication Date: 08/25/2016

Non-Publication Request: No

Early Publication Request: No

Title

TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/148,839

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	1 minus 20 =	*			OR	x 80 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 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).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1600

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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Page 9733

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/148,839	05/06/2016	Leonard BELL	AXJ-114BUSCN2

CONFIRMATION NO. 3159

133485
Nelson Mullins Riley & Scarborough LLP/
Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

INFORMAL NOTICE

OC000000082939290

Date Mailed: 05/17/2016

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Leonard BELL
  Russell P. ROTHER

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/tpetros/

PTO/AIA/01 (06-12)
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

As the below named inventor, I hereby declare that:

This declaration is directed to:
- [ ] The attached application, or
- [x] United States application or PCT international application number 15/148,839 filed on 05/06/2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Leonard Bell Date (Optional): _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

Doc code: Oath
Document Description: Oath or declaration filed

PTO/AIA/02 (07-13)
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SUBSTITUTE STATEMENT IN LIEU OF AN OATH OR DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (35 U.S.C. 115(d) AND 37 CFR 1.64)

Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

This statement is directed to:

[ ] The attached application,

OR

[X] United States application or PCT international application number 15/148,839 filed on 05/06/2016.

LEGAL NAME of inventor to whom this substitute statement applies:

(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

Russell P. ROTHER

Residence (except for a deceased or legally incapacitated inventor):

City	State	Country
Oklahoma City	OK	United States of America

Mailing Address (except for a deceased or legally incapacitated inventor):

10325 Forest Meadow Drive

City	State	Zip	Country
Oklahoma City	OK	73151	United States of America

I believe the above-named inventor or joint inventor to be the original inventor or an original joint inventor of a claimed invention in the application.

The above-identified application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this statement is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Relationship to the inventor to whom this substitute statement applies:

[ ] Legal Representative (for deceased or legally incapacitated inventor only),

[X] Assignee,

[ ] Person to whom the inventor is under an obligation to assign,

[ ] Person who otherwise shows a sufficient proprietary interest in the matter (petition under 37 CFR 1.46 is required), or

[ ] Joint Inventor.

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Page 9736

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SUBSTITUTE STATEMENT

Circumstances permitting execution of this substitute statement:

- [ ] Inventor is deceased,
- [ ] Inventor is under legal incapacity,
- [ ] Inventor cannot be found or reached after diligent effort, or
- [x] Inventor has refused to execute the oath or declaration under 37 CFR 1.63.

If there are joint inventors, please check the appropriate box below:

- [x] An application data sheet under 37 CFR 1.76 (PTO/AIA/14 or equivalent) naming the entire inventive entity has been or is currently submitted.

OR

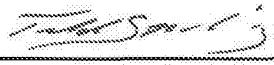
- [ ] An application data sheet under 37 CFR 1.76 (PTO/AIA/14 or equivalent) has not been submitted. Thus, a Substitute Statement Supplemental Sheet (PTO/AIA/11 or equivalent) naming the entire inventive entity and providing inventor information is attached. See 37 CFR 1.64(b).

WARNING:

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PERSON EXECUTING THIS SUBSTITUTE STATEMENT:

Name: Todd Spalding	Date (Optional):

Signature: 

APPLICANT NAME AND TITLE OF PERSON EXECUTING THIS SUBSTITUTE STATEMENT:

If the applicant is a juristic entity, list the applicant name and the title of the signer:

Applicant Name: Alexion Pharmaceuticals, Inc.

Title of Person Executing This Substitute Statement: Vice President, Head of Intellectual Property

The signer, whose title is supplied above, is authorized to act on behalf of the applicant.

Residence of the signer (unless provided in an application data sheet, PTO/AIA/14 or equivalent):

City	State	Country

Mailing Address of the signer (unless provided in an application data sheet, PTO/AIA/14 or equivalent)

100 College St.

City: New Haven	State: CT	Zip: 06510	Country: US

Note: Use an additional PTO/AIA/02 form for each inventor who is deceased, legally incapacitated, cannot be found or reached after diligent effort, or has refused to execute the oath or declaration under 37 CFR 1.63.

Electronic Acknowledgement Receipt

EFS ID:	25864926
Application Number:	15148839
International Application Number:	
Confirmation Number:	3159
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Danielle Gamache
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN2
Receipt Date:	24-MAY-2016
Filing Date:	06-MAY-2016
Time Stamp:	13:54:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	AXJ_114BUSCN2_Declarations.pdf	2627453 611f9dd6532afb737fd2b8b738fffb3c7782add2	no	3

Warnings:	
Information:	

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Page 9738

Total Files Size (in bytes):	2627453

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

Samsung Bioepis Exhibit No. 1002
Page 9739

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/148,839	05/06/2016	Leonard BELL	AXJ-114BUSCN2	3159

133485 7590 06/16/2016

Nelson Mullins Riley & Scarborough LLP/
Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
06/16/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

In re Application of
Leonard Bell et al.
Application No.: 15/148,839
Filed: 06 May 2016
Attorney Docket No.: AXJ-114BUSCN2
For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

: DECISION ON REQUEST TO
: PARTICIPATE IN THE PATENT
: PROSECUTION HIGHWAY
: PROGRAM AND PETITION
: TO MAKE SPECIAL UNDER
: 37 CFR 1.102(a)

This is a decision on the request to participate in the Patent Prosecution Highway (PPH) program and the petition under 37 CFR 1.102(a), filed 06 May 2016, to make the above-identified application special.

The request and petition are **GRANTED**.

DISCUSSION

A grantable request to participate in the PPH pilot program and petition to make special require:

1. The U.S. application for which participation in the Global/IP5 PPH pilot program is requested must have the same earliest date, whether this is the priority date or filing date, as that of a corresponding national or regional application filed with another Global/IP5 PPH participating office or a corresponding PCT international application for which one of the Global/IP5 PPH participating offices was the International Searching Authority (ISA) or the International Preliminary Examining Authority (IPEA).

2. Applicant must:
    a. Ensure all the claims in the U.S. application must sufficiently correspond or be amended to sufficiently correspond to the allowable/patentable claim(s) in the corresponding Office of Earlier Examination (OEE) application and
    b. Submit a claims correspondence table in English;
3. Examination of the U.S. application has not begun;
4. Applicant must submit:
    a. Documentation of prior office action:

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Page 9741

i. a copy of the office action(s) just prior to the "Decision to Grant a Patent" from each of the Global/IP5 PPH participating office application(s) containing the allowable/patentable claim(s) or
ii. if the allowable/patentable claims(s) are from a "Notification of Reasons for Refusal" then the Notification of Reasons for Refusal or
iii. if the Global/IP5 PPH participating office application is a first action allowance then no office action from the Global/IP5 PPH participating office is necessary should be indicated on the request/petition form or
iv. the latest work product in the international phase of the OEE PCT application;

b. An English language translation of the Global/IP5 PPH participating office action or work product from (4)(a)(i)-(ii) or (iv) above;

5. Applicant must submit:
   a. An IDS listing the documents cited by the Global/IP5 PPH participating office examiner in the Global/IP5 PPH participating office action or work product (unless already submitted in this application)
   b. Copies of the documents except U.S. patents or U.S. patent application publications (unless already submitted in this application);

The request to participate in the PPH pilot program and petition comply with the above requirements. Accordingly, the above-identified application has been accorded "special" status.

Telephone inquiries concerning this decision should be directed to JoAnne Burke at (571) 272-4584

All other inquiries concerning the examination or status of the application is accessible in the PAIR system at http://www.uspto.gov/ebc.index.html.

This application will be forwarded to the examiner for action on the merits commensurate with this decision once this application's formality reviews have been completed.

/JoAnne Burke/
JoAnne Burke
Paralegal Specialist
Office of Petitions

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Office of Petitions: Routing Sheet



Application No. 15/148,839

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

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Office of Petitions: Decision Count Sheet

Mailing Month

Application No. 15148839



For US serial numbers: enter number only, no slashes or commas. Ex: 10123456

For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: BURKE, JOANNE

Count (1) - Palm Credit 15/148,839

Decision: GRANT

FINANCE WORK NEEDED

☐ Select Check Box for YES



Decision Type: 652 - Petition to make special-PPH



Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED

☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED

☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

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Page 9744

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/148,839	05/06/2016	Leonard BELL	AXJ-114BUSCN2	3159

133485 7590 08/16/2016

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
08/16/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

Office Action Summary	Application No. 15/148,839	Applicant(s) BELL ET AL.	
	Examiner JAMES ROGERS	Art Unit 1644	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *5/6/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *5/6/2016*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

1. Claim 1 is pending and under consideration in the instant examination and reads on a compositions which is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution.

2. Applicant's IDSs, filed on 05/06/2016, are acknowledged and have been considered.

Priority

3. Applicant's claim for the benefit of a prior-filed application under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, or 365(c) is acknowledged. Applicant has not complied with one or more conditions for receiving the benefit of an earlier filing date under 35 U.S.C. 119(e) as follows:

The later-filed application must be an application for a patent for an invention which is also disclosed in the prior application (the parent or original nonprovisional application or provisional application). The disclosure of the invention in the parent application and in the later-filed application must be sufficient to comply with the requirements of 35 U.S.C. 112(a) or the first paragraph of 35 U.S.C. 112 (pre-AIA). See *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994).

The disclosure of the prior-filed application, US Provisional Application No. 60/783,070, fails to provide adequate support or enablement in the manner provided by 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph for one or more claims of this application. In particular, the claim recites a composition which is a 300 mg eculizumab single-use dosage comprising 30 ml of a 10 mg eculizumab/ml steril, preservative free solution.

Accordingly, the priority date of the instant claims of the current application is considered to be **3/15/2007**, the filing date of PCT/US2007/006606.

Claim Rejections - 35 USC § 103

4. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained even though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter

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pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

5. Claim 1 is rejected under 35 U.S.C. 103(a) as being unpatentable over Hillmen et al. (N Engl J Med 2004; 350: 552-9), in view of Evans et al. (US 6,355,245 B1, issued 3/12/2002), Wang (US 2005/0271660), as is evidenced by the parent specification of US Patent Application No. 13/426,973 at p. 16, lines 20-25.

Hillmen teaches a method for treating a human patient that suffers from paroxysmal nocturnal hemoglobinuria (PNH) by administering to the patient an effective amount of eculizumab (p. 552, "methods") which specifically binds to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b (p. 553, 3rd ¶). Hillmen teaches that patients received infusions of eculizumab weekly for four weeks, followed one week later by a 900 mg dose and then by a dose of 900 mg every other week through week 12 (p. 554 under “treatment schedule”).

The prior art references differ from the claimed invention in the recitation that the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml steril, preservative free solution.

However, Evans teaches antibody 5G1.1, which is the same as eculizumab (see parent specification US Patent Application No. 13/426,973, p. 16 lines 20-25), is effective at doses of 2.2, 3, or 5 mg per kg body weight; this corresponds to 154, 210, or 350 mg of antibody for a 70kg adult human (‘245 patent, column 16 lines 10-30). Evans also teaches that eculizumab is therapeutically effective when as little as 210 mg are present (column 16 lines 10-15). Evans teaches that the antibodies are particularly advantageous in that they are highly active even when administered in low doses (col 8 lines 25-32), providing a reasonable expectation of success in treating PHN with a dose less than what was disclosed by Hillmen. The reference teaches that it is within the skill of an artisan, particularly a physician, to determine the optimal dose (column 18 lines 15-30). Evans teaches formulations of the anti-C5 antibodies may be formulated in a pharmaceutically effective carrier such saline, which is considered to be a "preservative free solution". (col 18 lines 30-45).

However neither Hillmen nor '245 teaches a 300 mg single unit dosage as recited in the claims.

Nevertheless it would have been obvious to one of ordinary skill in the art to modify the teachings of Hillmen to administer 300 mg single unit dosages. Hillmen teaches that both 600 and 900 mg dosages can be used, and since Evans patent teaches that 210 or 350 mg of antibody would be expected to be therapeutically effective, arriving at the 300 mg dosage would be routine, convenient and obvious. One of ordinary skill in the art would have a reasonable expectation of success, as Evans teaches that the antibody exerts its effect at low doses.

Finally, Wang teaches that eculizumab is highly soluble, including at up to 30 mg per ml (paragraph 0171, Figure 10). Wang further teaches that pharmaceutically acceptable carriers include pyrogen-free water may be used in formulation of the antibody (¶71) (sterile, preservative free solution). Therefore the artisan of ordinary skill would have had a reasonable expectation of success in making a 10 mg / ml solution of the antibody sterile, preservative free solution.

From the combined teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

6. No claim is allowed.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to James Rogers whose telephone number is (571) 270-5374. The examiner can normally be reached Monday through Friday from 8:00 am to 5:30 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Daniel Kolker** can be reached on (571) 272-3181. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

August 9, 2016

/JAMES ROGERS/

Examiner, Art Unit 1644

/SHARON WEN/
Primary Examiner, Art Unit 1644

Samsung Bioepis Exhibit No. 1002
Page 9750

Notice of References Cited	Application/Control No. 15/148,839	Applicant(s)/Patent Under Reexamination BELL ET AL.	
	Examiner JAMES ROGERS	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-6,355,245 B1	03-2002	Evans; Mark J.	C07K14/472	424/130.1
*	B	US-2005/0271660 A1	12-2005	Wang, Yi	C07K16/18	424/144.1
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	hILLMEN ET AL. "eFFECT OF eCULIZUMAB ON hEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA" nejm 350(6), 2004, PP. 552-559
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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BIB DATA SHEET

CONFIRMATION NO. 3159

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/148,839	05/06/2016	424	1644	AXJ-114BUSCN2

APPLICANTS
Alexion Pharmaceuticals, Inc., New Haven, CT;

INVENTORS
Leonard BELL, Woodbridge, CT;
Russell P. ROTHER, Oklahoma City, OK;

**** CONTINUING DATA ***************************
This application is a CON of 13/426,973 03/22/2012
which is a CON of 12/225,040 05/13/2009 ABN
which is a 371 of PCT/US2007/006606 03/15/2007
which claims benefit of 60/783,070 03/15/2006

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
05/16/2016

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /JAMES L ROGERS/ Examiner's Signature	☐ Met after Allowance JR Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CT	4	1	1

ADDRESS
Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127
UNITED STATES

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

FILING FEE RECEIVED		
1740	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

BIB (Rev. 05/07).

Samsung Bioepis Exhibit No. 1002
Page 9752

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	
	Filing Date	
	First Named Inventor	Leonard BELL
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

U.S.PATENTS							Remove
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	1						
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	1							
If you wish to add additional Foreign Patent Document citation information please click the Add button								Add

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

EFS Web 2.1.17

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Leonard BELL
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

	1	Mortazavi et al., "The spectrum of PIG-A gene mutations in a plastic anemia/paroxysmal nocturnal hemoglobinuria (AAIPNH): a high incidence of multiple mutations and evidence of a mutational hot spot," Blood 101 2833-2841 (2003).	
	2	Moyo et al., "Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays," British J Haematology 126, 133-138 (2004).	
	3	Notice of Opposition for EP Patent No. 1720571, dated 3/18/2013.	
	4	Parker et al., Diagnosis and management of paroxysmal nocturnal hemoglobinuria, Blood, 106(12):3699-3709 (2005).	
	5	Paroxysmal nocturnal hemoglobinuria, included; PNH, included phosphatidylinositol glycan, class A, pseudogene 1, included; pigap1, included piga-related processed gene, included [online]. OM 1M-Online Medelian Inheritance in Man; John Hopkins University; [Retrieved on 2003-06-18]. Retrieved from the world wide web at ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=OMIM&dopt=detailed ...	
	6	Reiter et al., "Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease," Nature Medicine 8 (12):1383-1389 (2002).	
	7	Richards et al., "Evolution of GPI-Deficient Clones Predicts Clinical Course in Paroxysmal Nocturnal Haemoglobinuria," Blood, 104:Abstract 172 (2004).	
	8	Rodary et al., "Patient preference for either the EORTC QLQ-C30 or the FACIT Quality of Life (QOL) measures: a study performed in patient suffering from carcinoma of an unknown primary site (CUP)," European Journal of Cancer, Vol. 40, pp. 521-528 (2004).	
	9	Rosse et al., Immune-mediated hemolytic anemia, Hematology Am Soc Hematol Educ. Program, 48-62 (2004) XP002497825.	
	10	ROTHER et al., Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH), Molecular Immunology, Vol. 40(2-4), page 197; XP009163498, (2003).	
	11	Rother et al., The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin, JAMA 293 (13):1653-1662 (2005).	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Leonard BELL
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

	12	Stebler et al., High-dose recombinant human erythropoietin for treatment of anemia in myelodysplastic syndromes and paroxysmal nocturnal hemoglobinuria: a pilot study, Exp. Hematol. 18:1204-1208 (1990).	
	13	Strauss et al. "Alternative pathway of complement in sickle cell disease," Pediat. Res., vol. 11:285-289 (1977)	
	14	Stuart et al., "Sickle-cell disease," Lancet, vol. 364:1343-60 (2004)	
	15	Tang and Pikal, Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice, Pharmaceutical Research, Vol. 21 (2):191-200 (2004).	
	16	Thomas et al., Inhibition of complement activity by humanized anti-C5 antibody and single chain Fv, Mol.Imm. 33 (17-18):1389-1401, XP002262113 (1996).	
	17	Vakeva, et al., "Myocardial infarction and apoptosis after myocardial ischemia and reperfusion," Circulation 97:2259-2267 (1998).	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/JAMES L ROGERS/	Date Considered	08/08/2016

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Leonard BELL
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	AXJ-114BUSCN2

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2016-05-06
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60760

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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supplemental STN search

```
=> d hist

     (FILE 'HOME' ENTERED AT 15:48:46 ON 08 AUG 2016)

     FILE 'DISSABS, 1MOBILITY, AGRICOLA, BIOTECHNO, COMPENDEX, EMBASE, INSPEC,
     PASCAL, PQSCITECH, ADISCTI, ADISINSIGHT, ADISNEWS, ANABSTR, BIOSIS,
     BIOTECHDS, CABA, CAPLUS, CEABA-VTB, CIN, CROPB, CROPU, DDFB, DGENE,
     DRUGB, DRUGU, EMBAL, ESBIOBASE, FOMAD, ...' ENTERED AT 15:49:06 ON 08 AUG
     2016
L1             0 FILE DISSABS
L2             0 FILE 1MOBILITY
L3             0 FILE AGRICOLA
L4             0 FILE BIOTECHNO
L5             0 FILE COMPENDEX
L6           186 FILE EMBASE
L7             0 FILE INSPEC
L8             4 FILE PASCAL
L9             5 FILE PQSCITECH
L10           41 FILE ADISCTI
L11            1 FILE ADISINSIGHT
L12           32 FILE ADISNEWS
L13            0 FILE ANABSTR
L14          168 FILE BIOSIS
L15            0 FILE BIOTECHDS
L16            0 FILE CABA
L17           21 FILE CAPLUS
L18            0 FILE CEABA-VTB
L19            1 FILE CIN
L20            0 FILE CROPB
L21            0 FILE CROPU
L22            0 FILE DDFB
L23            0 FILE DGENE
L24            0 FILE DRUGB
L25           96 FILE DRUGU
L26            4 FILE EMBAL
L27           16 FILE ESBIOBASE
L28            0 FILE FOMAD
L29            0 FILE FROSTI
L30            0 FILE FSTA
L31            0 FILE GENBANK
L32           10 FILE IFIALL
L33            2 FILE IMSRESEARCH
L34            0 FILE KOSMET
L35           40 FILE MEDLINE
L36            0 FILE NTIS
L37            0 FILE PCTGEN
L38            0 FILE PS
L39            0 FILE RDISCLOSURE
L40           34 FILE SCISEARCH
L41           41 FILE TOXCENTER
L42            0 FILE USGENE
L43           39 FILE USPATFULL
L44            0 FILE USPATOLD
L45            7 FILE USPAT2
L46            0 FILE VETB
L47            0 FILE VETU
L48            7 FILE IPA
L49            0 FILE NAPRALERT
     TOTAL FOR ALL FILES
```


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supplemental STN search

```
L50         755 S (DOSE OR DOSAGE) (10A) (ECULIZUMAB OR SOLIRIS)
L51           0 FILE DISSABS
L52           0 FILE 1MOBILITY
L53           0 FILE AGRICOLA
L54           0 FILE BIOTECHNO
L55           0 FILE COMPENDEX
L56           0 FILE EMBASE
L57           0 FILE INSPEC
L58           0 FILE PASCAL
L59           0 FILE PQSCITECH
L60           0 FILE ADISCTI
L61           0 FILE ADISINSIGHT
L62           0 FILE ADISNEWS
L63           0 FILE ANABSTR
L64           0 FILE BIOSIS
L65           0 FILE BIOTECHDS
L66           0 FILE CABA
L67           0 FILE CAPLUS
L68           0 FILE CEABA-VTB
L69           0 FILE CIN
L70           0 FILE CROPB
L71           0 FILE CROPU
L72           0 FILE DDFB
L73           0 FILE DGENE
L74           0 FILE DRUGB
L75           0 FILE DRUGU
L76           0 FILE EMBAL
L77           0 FILE ESBIOBASE
L78           0 FILE FOMAD
L79           0 FILE FROSTI
L80           0 FILE FSTA
L81           0 FILE GENBANK
L82           0 FILE IFIALL
L83           0 FILE IMSRESEARCH
L84           0 FILE KOSMET
L85           0 FILE MEDLINE
L86           0 FILE NTIS
L87           0 FILE PCTGEN
L88           0 FILE PS
L89           0 FILE RDISCLOSURE
L90           0 FILE SCISEARCH
L91           0 FILE TOXCENTER
L92           0 FILE USGENE
L93           0 FILE USPATFULL
L94           0 FILE USPATOLD
L95           0 FILE USPAT2
L96           0 FILE VETB
L97           0 FILE VETU
L98           0 FILE IPA
L99           0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L100          0 S L50 (20A) (SINGLE (2A) UNIT)
L101          0 FILE DISSABS
L102          0 FILE 1MOBILITY
L103          0 FILE AGRICOLA
L104          0 FILE BIOTECHNO
L105          0 FILE COMPENDEX
L106          0 FILE EMBASE
L107          0 FILE INSPEC
```


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```
L108           0 FILE PASCAL
L109           0 FILE PQSCITECH
L110           0 FILE ADISCTI
L111           0 FILE ADISINSIGHT
L112           0 FILE ADISNEWS
L113           0 FILE ANABSTR
L114           0 FILE BIOSIS
L115           0 FILE BIOTECHDS
L116           0 FILE CABA
L117           0 FILE CAPLUS
L118           0 FILE CEABA-VTB
L119           0 FILE CIN
L120           0 FILE CROPB
L121           0 FILE CROPU
L122           0 FILE DDFB
L123           0 FILE DGENE
L124           0 FILE DRUGB
L125           0 FILE DRUGU
L126           0 FILE EMBAL
L127           0 FILE ESBIOBASE
L128           0 FILE FOMAD
L129           0 FILE FROSTI
L130           0 FILE FSTA
L131           0 FILE GENBANK
L132           0 FILE IFIALL
L133           0 FILE IMSRESEARCH
L134           0 FILE KOSMET
L135           0 FILE MEDLINE
L136           0 FILE NTIS
L137           0 FILE PCTGEN
L138           0 FILE PS
L139           0 FILE RDISCLOSURE
L140           0 FILE SCISEARCH
L141           0 FILE TOXCENTER
L142           0 FILE USGENE
L143           0 FILE USPATFULL
L144           0 FILE USPATOLD
L145           0 FILE USPAT2
L146           0 FILE VETB
L147           0 FILE VETU
L148           0 FILE IPA
L149           0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L150           0 S L50 (20A) (10 (2A) MG/ML)
L151           0 FILE DISSABS
L152           0 FILE 1MOBILITY
L153           0 FILE AGRICOLA
L154           0 FILE BIOTECHNO
L155           0 FILE COMPENDEX
L156           5 FILE EMBASE
L157           0 FILE INSPEC
L158           0 FILE PASCAL
L159           0 FILE PQSCITECH
L160           0 FILE ADISCTI
L161           0 FILE ADISINSIGHT
L162           0 FILE ADISNEWS
L163           0 FILE ANABSTR
L164           0 FILE BIOSIS
L165           0 FILE BIOTECHDS
```


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```
L166           0 FILE CABA
L167           1 FILE CAPLUS
L168           0 FILE CEABA-VTB
L169           0 FILE CIN
L170           0 FILE CROPB
L171           0 FILE CROPU
L172           0 FILE DDFB
L173           0 FILE DGENE
L174           0 FILE DRUGB
L175           6 FILE DRUGU
L176           0 FILE EMBAL
L177           1 FILE ESBIOBASE
L178           0 FILE FOMAD
L179           0 FILE FROSTI
L180           0 FILE FSTA
L181           0 FILE GENBANK
L182           1 FILE IFIALL
L183           0 FILE IMSRESEARCH
L184           0 FILE KOSMET
L185           1 FILE MEDLINE
L186           0 FILE NTIS
L187           0 FILE PCTGEN
L188           0 FILE PS
L189           0 FILE RDISCLOSURE
L190           1 FILE SCISEARCH
L191           0 FILE TOXCENTER
L192           0 FILE USGENE
L193           5 FILE USPATFULL
L194           0 FILE USPATOLD
L195           1 FILE USPAT2
L196           0 FILE VETB
L197           0 FILE VETU
L198           0 FILE IPA
L199           0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L200          22 S L50 (20A) (300 (2A) MG)
L201          15 DUP REM L200 (7 DUPLICATES REMOVED)
L202           0 FILE DISSABS
L203           0 FILE 1MOBILITY
L204           0 FILE AGRICOLA
L205           0 FILE BIOTECHNO
L206           0 FILE COMPENDEX
L207           0 FILE EMBASE
L208           0 FILE INSPEC
L209           0 FILE PASCAL
L210           0 FILE PQSCITECH
L211           0 FILE ADISCTI
L212           0 FILE ADISINSIGHT
L213           0 FILE ADISNEWS
L214           0 FILE ANABSTR
L215           0 FILE BIOSIS
L216           0 FILE BIOTECHDS
L217           0 FILE CABA
L218           0 FILE CAPLUS
L219           0 FILE CEABA-VTB
L220           0 FILE CIN
L221           0 FILE CROPB
L222           0 FILE CROPU
L223           0 FILE DDFB
```


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```
L224          0 FILE DGENE
L225          0 FILE DRUGB
L226          0 FILE DRUGU
L227          0 FILE EMBAL
L228          0 FILE ESBIOBASE
L229          0 FILE FOMAD
L230          0 FILE FROSTI
L231          0 FILE FSTA
L232          0 FILE GENBANK
L233          0 FILE IFIALL
L234          0 FILE IMSRESEARCH
L235          0 FILE KOSMET
L236          0 FILE MEDLINE
L237          0 FILE NTIS
L238          0 FILE PCTGEN
L239          0 FILE PS
L240          0 FILE RDISCLOSURE
L241          0 FILE SCISEARCH
L242          0 FILE TOXCENTER
L243          0 FILE USGENE
L244          3 FILE USPATFULL
L245          0 FILE USPATOLD
L246          0 FILE USPAT2
L247          0 FILE VETB
L248          0 FILE VETU
L249          0 FILE IPA
L250          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L251          3 S L50 AND (SINGLE (2A) UNIT)
L252          3 DUP REM L251 (0 DUPLICATES REMOVED)
L253          0 FILE DISSABS
L254          0 FILE 1MOBILITY
L255          0 FILE AGRICOLA
L256          0 FILE BIOTECHNO
L257          0 FILE COMPENDEX
L258          8 FILE EMBASE
L259          0 FILE INSPEC
L260          0 FILE PASCAL
L261          0 FILE PQSCITECH
L262          0 FILE ADISCTI
L263          0 FILE ADISINSIGHT
L264          0 FILE ADISNEWS
L265          0 FILE ANABSTR
L266          1 FILE BIOSIS
L267          0 FILE BIOTECHDS
L268          0 FILE CABA
L269          1 FILE CAPLUS
L270          0 FILE CEABA-VTB
L271          0 FILE CIN
L272          0 FILE CROPB
L273          0 FILE CROPU
L274          0 FILE DDFB
L275          0 FILE DGENE
L276          0 FILE DRUGB
L277          8 FILE DRUGU
L278          1 FILE EMBAL
L279          1 FILE ESBIOBASE
L280          0 FILE FOMAD
L281          0 FILE FROSTI
```


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```
L282          0 FILE FSTA
L283          0 FILE GENBANK
L284          1 FILE IFIALL
L285          0 FILE IMSRESEARCH
L286          0 FILE KOSMET
L287          2 FILE MEDLINE
L288          0 FILE NTIS
L289          0 FILE PCTGEN
L290          0 FILE PS
L291          0 FILE RDISCLOSURE
L292          2 FILE SCISEARCH
L293          0 FILE TOXCENTER
L294          0 FILE USGENE
L295         12 FILE USPATFULL
L296          0 FILE USPATOLD
L297          1 FILE USPAT2
L298          0 FILE VETB
L299          0 FILE VETU
L300          0 FILE IPA
L301          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L302         38 S L50 AND (300 (2A) MG)
L303          0 FILE DISSABS
L304          0 FILE 1MOBILITY
L305          0 FILE AGRICOLA
L306          0 FILE BIOTECHNO
L307          0 FILE COMPENDEX
L308          0 FILE EMBASE
L309          0 FILE INSPEC
L310          0 FILE PASCAL
L311          0 FILE PQSCITECH
L312          0 FILE ADISCTI
L313          0 FILE ADISINSIGHT
L314          0 FILE ADISNEWS
L315          0 FILE ANABSTR
L316          0 FILE BIOSIS
L317          0 FILE BIOTECHDS
L318          0 FILE CABA
L319          0 FILE CAPLUS
L320          0 FILE CEABA-VTB
L321          0 FILE CIN
L322          0 FILE CROPB
L323          0 FILE CROPU
L324          0 FILE DDFB
L325          0 FILE DGENE
L326          0 FILE DRUGB
L327          0 FILE DRUGU
L328          0 FILE EMBAL
L329          0 FILE ESBIOBASE
L330          0 FILE FOMAD
L331          0 FILE FROSTI
L332          0 FILE FSTA
L333          0 FILE GENBANK
L334          0 FILE IFIALL
L335          0 FILE IMSRESEARCH
L336          0 FILE KOSMET
L337          0 FILE MEDLINE
L338          0 FILE NTIS
L339          0 FILE PCTGEN
```


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```
L340            0 FILE PS
L341            0 FILE RDISCLOSURE
L342            0 FILE SCISEARCH
L343            0 FILE TOXCENTER
L344            0 FILE USGENE
L345            0 FILE USPATFULL
L346            0 FILE USPATOLD
L347            0 FILE USPAT2
L348            0 FILE VETB
L349            0 FILE VETU
L350            0 FILE IPA
L351            0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L352            0 S L302 AND (10 (2A) MG/ML)
L353            0 FILE DISSABS
L354            0 FILE 1MOBILITY
L355            0 FILE AGRICOLA
L356            0 FILE BIOTECHNO
L357            0 FILE COMPENDEX
L358            0 FILE EMBASE
L359            0 FILE INSPEC
L360            0 FILE PASCAL
L361            0 FILE PQSCITECH
L362            0 FILE ADISCTI
L363            0 FILE ADISINSIGHT
L364            0 FILE ADISNEWS
L365            0 FILE ANABSTR
L366            0 FILE BIOSIS
L367            0 FILE BIOTECHDS
L368            0 FILE CABA
L369            0 FILE CAPLUS
L370            0 FILE CEABA-VTB
L371            0 FILE CIN
L372            0 FILE CROPB
L373            0 FILE CROPU
L374            0 FILE DDFB
L375            0 FILE DGENE
L376            0 FILE DRUGB
L377            0 FILE DRUGU
L378            0 FILE EMBAL
L379            0 FILE ESBIOBASE
L380            0 FILE FOMAD
L381            0 FILE FROSTI
L382            0 FILE FSTA
L383            0 FILE GENBANK
L384            0 FILE IFIALL
L385            0 FILE IMSRESEARCH
L386            0 FILE KOSMET
L387            0 FILE MEDLINE
L388            0 FILE NTIS
L389            0 FILE PCTGEN
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L392            0 FILE SCISEARCH
L393            0 FILE TOXCENTER
L394            0 FILE USGENE
L395            0 FILE USPATFULL
L396            0 FILE USPATOLD
L397            0 FILE USPAT2
```


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```
L398          0 FILE VETB
L399          0 FILE VETU
L400          0 FILE IPA
L401          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L402          0 S L352 AND PY<2008
L403         19 FILE DISSABS
L404          0 FILE 1MOBILITY
L405         13 FILE AGRICOLA
L406        176 FILE BIOTECHNO
L407          9 FILE COMPENDEX
L408        977 FILE EMBASE
L409          1 FILE INSPEC
L410        146 FILE PASCAL
L411        212 FILE PQSCITECH
L412         15 FILE ADISCTI
L413         18 FILE ADISINSIGHT
L414         11 FILE ADISNEWS
L415          4 FILE ANABSTR
L416        619 FILE BIOSIS
L417         71 FILE BIOTECHDS
L418         45 FILE CABA
L419        786 FILE CAPLUS
L420          6 FILE CEABA-VTB
L421         35 FILE CIN
L422          0 FILE CROPB
L423          0 FILE CROPU
L424          4 FILE DDFB
L425       2828 FILE DGENE
L426          4 FILE DRUGB
L427         71 FILE DRUGU
L428          7 FILE EMBAL
L429        235 FILE ESBIOBASE
L430          0 FILE FOMAD
L431          0 FILE FROSTI
L432          2 FILE FSTA
L433       1292 FILE GENBANK
L434        277 FILE IFIALL
L435          8 FILE IMSRESEARCH
L436          0 FILE KOSMET
L437        601 FILE MEDLINE
L438          4 FILE NTIS
L439        864 FILE PCTGEN
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L441          0 FILE RDISCLOSURE
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L443        376 FILE TOXCENTER
L444       3290 FILE USGENE
L445       1467 FILE USPATFULL
L446          1 FILE USPATOLD
L447        554 FILE USPAT2
L448          0 FILE VETB
L449          1 FILE VETU
L450         17 FILE IPA
L451          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L452      15589 S (C5 (10A) ANTIBOD?)
L453          0 FILE DISSABS
L454          0 FILE 1MOBILITY
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```
L455          0 FILE AGRICOLA
L456          0 FILE BIOTECHNO
L457          0 FILE COMPENDEX
L458          8 FILE EMBASE
L459          0 FILE INSPEC
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L461          0 FILE PQSCITECH
L462          1 FILE ADISCTI
L463          0 FILE ADISINSIGHT
L464          0 FILE ADISNEWS
L465          0 FILE ANABSTR
L466          1 FILE BIOSIS
L467          0 FILE BIOTECHDS
L468          0 FILE CABA
L469          0 FILE CAPLUS
L470          0 FILE CEABA-VTB
L471          0 FILE CIN
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L473          0 FILE CROPU
L474          0 FILE DDFB
L475          0 FILE DGENE
L476          0 FILE DRUGB
L477          1 FILE DRUGU
L478          0 FILE EMBAL
L479          0 FILE ESBIOBASE
L480          0 FILE FOMAD
L481          0 FILE FROSTI
L482          0 FILE FSTA
L483          0 FILE GENBANK
L484          2 FILE IFIALL
L485          0 FILE IMSRESEARCH
L486          0 FILE KOSMET
L487          1 FILE MEDLINE
L488          0 FILE NTIS
L489          0 FILE PCTGEN
L490          0 FILE PS
L491          0 FILE RDISCLOSURE
L492          1 FILE SCISEARCH
L493          0 FILE TOXCENTER
L494          0 FILE USGENE
L495        310 FILE USPATFULL
L496          0 FILE USPATOLD
L497        105 FILE USPAT2
L498          0 FILE VETB
L499          0 FILE VETU
L500          0 FILE IPA
L501          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L502        430 S L452 AND (300 (2A) MG)
L503          0 FILE DISSABS
L504          0 FILE 1MOBILITY
L505          0 FILE AGRICOLA
L506          0 FILE BIOTECHNO
L507          0 FILE COMPENDEX
L508          0 FILE EMBASE
L509          0 FILE INSPEC
L510          0 FILE PASCAL
L511          0 FILE PQSCITECH
L512          0 FILE ADISCTI
```


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```
L513            0 FILE ADISINSIGHT
L514            0 FILE ADISNEWS
L515            0 FILE ANABSTR
L516            0 FILE BIOSIS
L517            0 FILE BIOTECHDS
L518            0 FILE CABA
L519            0 FILE CAPLUS
L520            0 FILE CEABA-VTB
L521            0 FILE CIN
L522            0 FILE CROPB
L523            0 FILE CROPU
L524            0 FILE DDFB
L525            0 FILE DGENE
L526            0 FILE DRUGB
L527            0 FILE DRUGU
L528            0 FILE EMBAL
L529            0 FILE ESBIOBASE
L530            0 FILE FOMAD
L531            0 FILE FROSTI
L532            0 FILE FSTA
L533            0 FILE GENBANK
L534            0 FILE IFIALL
L535            0 FILE IMSRESEARCH
L536            0 FILE KOSMET
L537            0 FILE MEDLINE
L538            0 FILE NTIS
L539            0 FILE PCTGEN
L540            0 FILE PS
L541            0 FILE RDISCLOSURE
L542            0 FILE SCISEARCH
L543            0 FILE TOXCENTER
L544            0 FILE USGENE
L545            0 FILE USPATFULL
L546            0 FILE USPATOLD
L547            0 FILE USPAT2
L548            0 FILE VETB
L549            0 FILE VETU
L550            0 FILE IPA
L551            0 FILE NAPRALERT
      TOTAL FOR ALL FILES
L552            0 S L502 AND (10 (2A) MG/ML)
L553            0 FILE DISSABS
L554            0 FILE 1MOBILITY
L555            0 FILE AGRICOLA
L556            0 FILE BIOTECHNO
L557            0 FILE COMPENDEX
L558            0 FILE EMBASE
L559            0 FILE INSPEC
L560            0 FILE PASCAL
L561            0 FILE PQSCITECH
L562            0 FILE ADISCTI
L563            0 FILE ADISINSIGHT
L564            0 FILE ADISNEWS
L565            0 FILE ANABSTR
L566            0 FILE BIOSIS
L567            0 FILE BIOTECHDS
L568            0 FILE CABA
L569            0 FILE CAPLUS
L570            0 FILE CEABA-VTB
```


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```
L571          0 FILE CIN
L572          0 FILE CROPB
L573          0 FILE CROPU
L574          0 FILE DDFB
L575          0 FILE DGENE
L576          0 FILE DRUGB
L577          0 FILE DRUGU
L578          0 FILE EMBAL
L579          0 FILE ESBIOBASE
L580          0 FILE FOMAD
L581          0 FILE FROSTI
L582          0 FILE FSTA
L583          0 FILE GENBANK
L584          1 FILE IFIALL
L585          0 FILE IMSRESEARCH
L586          0 FILE KOSMET
L587          0 FILE MEDLINE
L588          0 FILE NTIS
L589          0 FILE PCTGEN
L590          0 FILE PS
L591          0 FILE RDISCLOSURE
L592          0 FILE SCISEARCH
L593          0 FILE TOXCENTER
L594          0 FILE USGENE
L595          2 FILE USPATFULL
L596          0 FILE USPATOLD
L597          0 FILE USPAT2
L598          0 FILE VETB
L599          0 FILE VETU
L600          0 FILE IPA
L601          0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L602          3 S L452 (20A) (SINGLE (2A) UNIT)
L603          2 DUP REM L602 (1 DUPLICATE REMOVED)
L604          0 FILE DISSABS
L605          0 FILE 1MOBILITY
L606          0 FILE AGRICOLA
L607          0 FILE BIOTECHNO
L608          0 FILE COMPENDEX
L609          1 FILE EMBASE
L610          0 FILE INSPEC
L611          0 FILE PASCAL
L612          0 FILE PQSCITECH
L613          0 FILE ADISCTI
L614          0 FILE ADISINSIGHT
L615          0 FILE ADISNEWS
L616          0 FILE ANABSTR
L617          0 FILE BIOSIS
L618          0 FILE BIOTECHDS
L619          0 FILE CABA
L620          0 FILE CAPLUS
L621          0 FILE CEABA-VTB
L622          0 FILE CIN
L623          0 FILE CROPB
L624          0 FILE CROPU
L625          0 FILE DDFB
L626          0 FILE DGENE
L627          0 FILE DRUGB
L628          1 FILE DRUGU
```


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```
L629           0 FILE EMBAL
L630           0 FILE ESBIOBASE
L631           0 FILE FOMAD
L632           0 FILE FROSTI
L633           0 FILE FSTA
L634           0 FILE GENBANK
L635           2 FILE IFIALL
L636           0 FILE IMSRESEARCH
L637           0 FILE KOSMET
L638           0 FILE MEDLINE
L639           0 FILE NTIS
L640           0 FILE PCTGEN
L641           0 FILE PS
L642           0 FILE RDISCLOSURE
L643           0 FILE SCISEARCH
L644           0 FILE TOXCENTER
L645           0 FILE USGENE
L646           5 FILE USPATFULL
L647           0 FILE USPATOLD
L648           0 FILE USPAT2
L649           0 FILE VETB
L650           0 FILE VETU
L651           0 FILE IPA
L652           0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L653           9 S L452 (20A) (300 (2A) MG)
L654           6 DUP REM L653 (3 DUPLICATES REMOVED)
L655           0 FILE DISSABS
L656           0 FILE 1MOBILITY
L657           0 FILE AGRICOLA
L658           0 FILE BIOTECHNO
L659           0 FILE COMPENDEX
L660          16 FILE EMBASE
L661           0 FILE INSPEC
L662           1 FILE PASCAL
L663           0 FILE PQSCITECH
L664           1 FILE ADISCTI
L665           0 FILE ADISINSIGHT
L666           0 FILE ADISNEWS
L667           0 FILE ANABSTR
L668           2 FILE BIOSIS
L669           0 FILE BIOTECHDS
L670           0 FILE CABA
L671           2 FILE CAPLUS
L672           0 FILE CEABA-VTB
L673           0 FILE CIN
L674           0 FILE CROPB
L675           0 FILE CROPU
L676           0 FILE DDFB
L677           0 FILE DGENE
L678           0 FILE DRUGB
L679          11 FILE DRUGU
L680           0 FILE EMBAL
L681           1 FILE ESBIOBASE
L682           0 FILE FOMAD
L683           0 FILE FROSTI
L684           0 FILE FSTA
L685           0 FILE GENBANK
L686           1 FILE IFIALL
```


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```
L687           0 FILE IMSRESEARCH
L688           0 FILE KOSMET
L689           3 FILE MEDLINE
L690           0 FILE NTIS
L691           0 FILE PCTGEN
L692           0 FILE PS
L693           0 FILE RDISCLOSURE
L694           3 FILE SCISEARCH
L695           1 FILE TOXCENTER
L696           0 FILE USGENE
L697           7 FILE USPATFULL
L698           0 FILE USPATOLD
L699           1 FILE USPAT2
L700           0 FILE VETB
L701           0 FILE VETU
L702           0 FILE IPA
L703           0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L704          50 S (300 (2A) MG) (10A) (ECULIZUMAB OR SOLIRIS)
L705          34 DUP REM L704 (16 DUPLICATES REMOVED)
L706           0 S L705
L707           0 FILE DISSABS
L708           0 S L705
L709           0 FILE 1MOBILITY
L710           0 S L705
L711           0 FILE AGRICOLA
L712           0 S L705
L713           0 FILE BIOTECHNO
L714           0 S L705
L715           0 FILE COMPENDEX
L716          16 S L705
L717           0 FILE EMBASE
L718           0 S L705
L719           0 FILE INSPEC
L720           0 S L705
L721           0 FILE PASCAL
L722           0 S L705
L723           0 FILE PQSCITECH
L724           1 S L705
L725           0 FILE ADISCTI
L726           0 S L705
L727           0 FILE ADISINSIGHT
L728           0 S L705
L729           0 FILE ADISNEWS
L730           0 S L705
L731           0 FILE ANABSTR
L732           2 S L705
L733           0 FILE BIOSIS
L734           0 S L705
L735           0 FILE BIOTECHDS
L736           0 S L705
L737           0 FILE CABA
L738           0 S L705
L739           0 FILE CAPLUS
L740           0 S L705
L741           0 FILE CEABA-VTB
L742           0 S L705
L743           0 FILE CIN
L744           0 S L705
```


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```
L745          0 FILE CROPB
L746          0 S L705
L747          0 FILE CROPU
L748          0 S L705
L749          0 FILE DDFB
L750          0 S L705
L751          0 FILE DGENE
L752          0 S L705
L753          0 FILE DRUGB
L754          7 S L705
L755          0 FILE DRUGU
L756          0 S L705
L757          0 FILE EMBAL
L758          0 S L705
L759          0 FILE ESBIOBASE
L760          0 S L705
L761          0 FILE FOMAD
L762          0 S L705
L763          0 FILE FROSTI
L764          0 S L705
L765          0 FILE FSTA
L766          0 S L705
L767          0 FILE GENBANK
L768          1 S L705
L769          0 FILE IFIALL
L770          0 S L705
L771          0 FILE IMSRESEARCH
L772          0 S L705
L773          0 FILE KOSMET
L774          0 S L705
L775          0 FILE MEDLINE
L776          0 S L705
L777          0 FILE NTIS
L778          0 S L705
L779          0 FILE PCTGEN
L780          0 S L705
L781          0 FILE PS
L782          0 S L705
L783          0 FILE RDISCLOSURE
L784          0 S L705
L785          0 FILE SCISEARCH
L786          0 S L705
L787          0 FILE TOXCENTER
L788          0 S L705
L789          0 FILE USGENE
L790          6 S L705
L791          0 FILE USPATFULL
L792          0 S L705
L793          0 FILE USPATOLD
L794          1 S L705
L795          0 FILE USPAT2
L796          0 S L705
L797          0 FILE VETB
L798          0 S L705
L799          0 FILE VETU
L800          0 S L705
L801          0 FILE IPA
L802          0 S L705
L803          0 FILE NAPRALERT
```


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```
     TOTAL FOR ALL FILES
L804           0 S L705 AND (10 (2A) MG/ML)
L805           0 S L705
L806           0 FILE DISSABS
L807           0 S L705
L808           0 FILE 1MOBILITY
L809           0 S L705
L810           0 FILE AGRICOLA
L811           0 S L705
L812           0 FILE BIOTECHNO
L813           0 S L705
L814           0 FILE COMPENDEX
L815          16 S L705
L816           0 FILE EMBASE
L817           0 S L705
L818           0 FILE INSPEC
L819           0 S L705
L820           0 FILE PASCAL
L821           0 S L705
L822           0 FILE PQSCITECH
L823           1 S L705
L824           0 FILE ADISCTI
L825           0 S L705
L826           0 FILE ADISINSIGHT
L827           0 S L705
L828           0 FILE ADISNEWS
L829           0 S L705
L830           0 FILE ANABSTR
L831           2 S L705
L832           0 FILE BIOSIS
L833           0 S L705
L834           0 FILE BIOTECHDS
L835           0 S L705
L836           0 FILE CABA
L837           0 S L705
L838           0 FILE CAPLUS
L839           0 S L705
L840           0 FILE CEABA-VTB
L841           0 S L705
L842           0 FILE CIN
L843           0 S L705
L844           0 FILE CROPB
L845           0 S L705
L846           0 FILE CROPU
L847           0 S L705
L848           0 FILE DDFB
L849           0 S L705
L850           0 FILE DGENE
L851           0 S L705
L852           0 FILE DRUGB
L853           7 S L705
L854           0 FILE DRUGU
L855           0 S L705
L856           0 FILE EMBAL
L857           0 S L705
L858           0 FILE ESBIOBASE
L859           0 S L705
L860           0 FILE FOMAD
L861           0 S L705
```


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```
L862            0 FILE FROSTI
L863            0 S L705
L864            0 FILE FSTA
L865            0 S L705
L866            0 FILE GENBANK
L867            1 S L705
L868            0 FILE IFIALL
L869            0 S L705
L870            0 FILE IMSRESEARCH
L871            0 S L705
L872            0 FILE KOSMET
L873            0 S L705
L874            0 FILE MEDLINE
L875            0 S L705
L876            0 FILE NTIS
L877            0 S L705
L878            0 FILE PCTGEN
L879            0 S L705
L880            0 FILE PS
L881            0 S L705
L882            0 FILE RDISCLOSURE
L883            0 S L705
L884            0 FILE SCISEARCH
L885            0 S L705
L886            0 FILE TOXCENTER
L887            0 S L705
L888            0 FILE USGENE
L889            6 S L705
L890            0 FILE USPATFULL
L891            0 S L705
L892            0 FILE USPATOLD
L893            1 S L705
L894            0 FILE USPAT2
L895            0 S L705
L896            0 FILE VETB
L897            0 S L705
L898            0 FILE VETU
L899            0 S L705
L900            0 FILE IPA
L901            0 S L705
L902            0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L903            0 S L705 AND PY<2008
L904            0 FILE DISSABS
L905            0 FILE 1MOBILITY
L906            0 FILE AGRICOLA
L907            0 FILE BIOTECHNO
L908            0 FILE COMPENDEX
L909            1 FILE EMBASE
L910            0 FILE INSPEC
L911            0 FILE PASCAL
L912            0 FILE PQSCITECH
L913            0 FILE ADISCTI
L914            0 FILE ADISINSIGHT
L915            0 FILE ADISNEWS
L916            0 FILE ANABSTR
L917            0 FILE BIOSIS
L918            0 FILE BIOTECHDS
L919            0 FILE CABA
```


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```
L920            0 FILE CAPLUS
L921            0 FILE CEABA-VTB
L922            0 FILE CIN
L923            0 FILE CROPB
L924            0 FILE CROPU
L925            0 FILE DDFB
L926            0 FILE DGENE
L927            0 FILE DRUGB
L928            1 FILE DRUGU
L929            0 FILE EMBAL
L930            0 FILE ESBIOBASE
L931            0 FILE FOMAD
L932            0 FILE FROSTI
L933            0 FILE FSTA
L934            0 FILE GENBANK
L935            1 FILE IFIALL
L936            0 FILE IMSRESEARCH
L937            0 FILE KOSMET
L938            0 FILE MEDLINE
L939            0 FILE NTIS
L940            0 FILE PCTGEN
L941            0 FILE PS
L942            0 FILE RDISCLOSURE
L943            0 FILE SCISEARCH
L944            0 FILE TOXCENTER
L945            0 FILE USGENE
L946            3 FILE USPATFULL
L947            0 FILE USPATOLD
L948            0 FILE USPAT2
L949            0 FILE VETB
L950            0 FILE VETU
L951            0 FILE IPA
L952            0 FILE NAPRALERT
     TOTAL FOR ALL FILES
L953            6 S (300 (2A) MG) (10A) (C5 (10A) ANTIBOD?)
L954            4 DUP REM L953 (2 DUPLICATES REMOVED)
```


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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: May 6, 2016
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.: Not Yet Assigned

Confirmation No.: N/A

Filed: Concurrently Herewith

Art Unit: N/A

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

Examiner: Not Yet Assigned

INFORMATION DISCLOSURE STATEMENT (IDS)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In compliance with 37 C.F.R. § 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached PTO/SB/08.

For the Examiner's convenience in reviewing this continuation application, Applicant submits a consolidated PTO Form SB/08, listing all references cited during the prosecution of the parent application. The present application is a continuation of U.S. Application No. 13/426,973, filed March 22, 2012 (Atty. Docket No. AXJ-114BUSCN). In accordance with 37 C.F.R. §1.98(d), copies of the references previously cited by or submitted to the Office in the parent application are not enclosed, but will be provided upon request. In accordance with 37 C.F.R. § 1.98(a)(2)(i)-(iv), Applicant submits herewith copies of a non-patent literature references.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

It is respectfully requested that the documents listed on the PTO/SB/08 be expressly considered by the Examiner during the prosecution of this application, and that the documents be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

Applicants call to the attention of the Examiner the following Applications and Office Actions cited therein:

Applications				
Examiner's Initials	*Serial No.*	*Filing Date*	*First Named Inventor*	*Docket No.*
	13/426,973	03-22-2012	Leonard Bell	AXJ-114BUSCN
	12/225,040	05-13-2009	Leonard Bell	AXJ-114BUS
	12/621,792	11-19-2009	Leonard Bell	AXJ-114DV
	10/771,552	02-03-2004	Leonard Bell	AXJ-114RCE
	11/050,543	02-03-2005	Leonard Bell	AXJ-114CPRCE
	11/595,118	11-08-2006	Leonard Bell	AXJ-114CP2
	12/902,617	10-12-2010	Leonard Bell	AXJ-114CPCN
	13/335,518	12-22-2011	Leonard Bell	AXJ-114CPCN2RCE

Office Actions (copies enclosed)			
Examiner's Initials	Serial No.	Date Mailed from USPTO	Examiner
	13/426,973	01-27-2014	J. Rogers
	13/426,973	05-31-2013	J. Rogers
	13/426,973	08-20-2012	J. Rogers
	12/225,040	09-22-2011	J. Rogers
	12/225,040	06-01-2011	J. Rogers
	12/225,040	04-11-2011	J. Rogers
	12/621,792	09-30-2010	P. Gambel
	10/771,552	12-22-2008	F. Vandervegt
	10/771,552	03-21-2008	F. Vandervegt
	10/771,552	08-10-2007	F. Vandervegt
	10/771,552	02-26-2007	F. Vandervegt
	10/771,552	09-26-2006	F. Vandervegt
	11/050,543	07-09-2010	P. Gambel
	11/050,543	04-02-2008	F. Vandervegt
	11/050,543	10-10-2007	F. Vandervegt
	11/050,543	06-11-2007	F. Vandervegt
	11/595,118	01-21-2011	P. Gambel
	11/595,118	10-05-2010	P. Gambel

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

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	11/595,118	07-07-2010	P. Gambel
	11/595,118	04-03-2009	F. Vandervegt
	12/902,617	06-23-2011	P. Gambel
	12/902,617	04-04-2011	P. Gambel
	13/335,518	09-12-2014	P. Gambel
	13/335,518	02-06-2014	P. Gambel
	13/335,518	11-07-2013	P. Gambel

/JAMES L ROGERS/ 08/08/2016

The filing of this Information Disclosure Statement is not to be interpreted as a representation that the cited documents are material, that an exhaustive search has been conducted, or that no other relevant information exists. Nor shall the citation of any documents herein be construed *per se* as a representation that such document is prior art. Moreover, Applicant understands the Examiner will make an independent evaluation of the cited documents.

This Information Disclosure Statement accompanies the new patent application submitted herewith. Accordingly, no fees are due with this submission (37 C.F.R § 1.97(b)(3)). However, the Director is hereby authorized to charge any fee deficiency which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2.

Dated: May 6, 2016

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY &
SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L.R/

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15148839	BELL ET AL.
	Examiner	Art Unit
	JAMES ROGERS	1644

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
STN: DISSABS MEETINGS BIOSCI CAPLUS MEDICINE	8/8/2016	JR
EAST: PGPUB, USPAT, JPO, EPO, INVENTOR SEARCH	8/8/2016	JR

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/James Rogers/ Examiner Art Unit 1644

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	33	("300" near2 mg) near10 (eculizumab or soliris)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:25
L2	0	(61/199764).APP.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 15:34
L3	0	(61/199764).APP.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 15:34
L4	0	(61/199562).APP.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 15:34
L5	13	("10" near2 mg/ml) near10 (eculizumab or soliris)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:37
L6	4	(C5 near10 antibody) near10 ("300" near2 mg)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:40
L7	3	(C5 near10 antibody) near10 ("10" near2 mg/ml)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:42
L8	159	(leonard near3 bell).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:43
L9	352	(russell near3 rother).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:43
L10	1	L8 and (("300" near2 mg) near10 (eculizumab or soliris))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:44
L11	1	L9 and (("300" near2 mg) near10 (eculizumab or soliris))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 15:46
S1	0	((((III near2 A) near3 receptor) near5 V158) near10 (support or resin or column or chromatography)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 09:35
S2	0	((gamma near2 Fc) near5 V158) near10 (support or resin or column or chromatography)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 09:37
S3	0	((gamma near3 RIIIa) near5 V158) near10 (support or resin or	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	ON	2016/08/08 09:37

		column or chromatography)	JPO; DERWENT; IBM_TDB			
S4	0	(FCGR3A near5 V158) near10 (support or resin or column or chromatography)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 09:37
S5	0	(CD16a near5 V158) near10 (support or resin or column or chromatography)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 09:38
S6	0	(CD-16a near5 V158) near10 (support or resin or column or chromatography)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2016/08/08 09:38
S7	1	("9353187").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 09:53
S8	1	("20150079082").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 10:10
S9	1	("7317091").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 10:14
S10	1	("20140255399").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/08/08 10:22

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S11	0	("((IIInear2A)near3receptor).clm.").PN.	US-PGPUB; USPAT	OR	OFF	2016/08/08 09:12
S12	7	((III near2 A) near3 receptor).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:13
S13	226	(gamma near2 Fc).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:14
S14	3	(gamma near3 RIIIa).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:14
S15	96	(FCGR3A).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:15
S16	62	(CD16a).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:15
S17	0	(CD-16a).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:15
S18	3	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S12	US-PGPUB; USPAT	OR	ON	2016/08/08 09:16
S19	110	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S13	US-PGPUB; USPAT	OR	ON	2016/08/08 09:17
S20	91	S19 and (antibody or antibodies or IgG).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:18

S21	110	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S13	US-PGPUB; USPAT	OR	ON	2016/08/08 09:25
S22	2	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S14	US-PGPUB; USPAT	OR	ON	2016/08/08 09:25
S23	45	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S15	US-PGPUB; USPAT	OR	ON	2016/08/08 09:25
S24	17	(purified of purification or purifying or isolate or isolating or isolated Or separate or separated or separating).clm. and S16	US-PGPUB; USPAT	OR	ON	2016/08/08 09:26
S25	8	S21 and (support or resin or column or chromatography).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:26
S26	1	S22 and (support or resin or column or chromatography).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:26
S27	0	((((III near2 A) near3 receptor) near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:30
S28	1	((gamma near2 Fc)near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:31
S29	1	((gamma near3 RIIIa) near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:32
S30	1	(FCGR3A near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:33
S31	0	(CD16a near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:33
S32	0	(CD-16a near5 V158).clm.	US-PGPUB; USPAT	OR	ON	2016/08/08 09:34

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned
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	1	4686100		1987-08-11	Raffin et al.	
	2	5135916		1992-08-04	Sims et al.	
	3	5660825		1997-08-26	Sims et al.	
	4	6355245		2002-03-12	Evans et al.	
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	1	20030049683	A1	2003-03-13	Bowdish et al.	
	2	20050191298	A1	2005-09-01	Bell et al.	
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	4	20050169921	A1	2005-08-04	Bell et al.	
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	6	20070116710	A1	2007-05-24	Bell et al.	
	7	20090220508	A1	2009-09-03	Bell et al.	
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	9	20110086040	A1	2011-04-14	Bell et al.	
	10	20120308559	A1	2012-12-06	Bell et al.	
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	1	0411306	EP	A1	1991-02-06	Goetze, Otto Prof. Dr. Med		
	2	2004/029207	WO	A2	2004-04-08	Xencor Inc		
	3	2005074607	WO	A2	2005-08-18	Alexion Pharmaceuticals, Inc		

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	1	Alexion Announces Presentation of Preliminary Results from Phase I Pilot Study of Eculizumab Treatment of Patients with Paroxysmal Nocturnal Hemoglobinuria"; [online]; Alexion Pharmaceuticals: News; [Retrieved on 2003-06-18]. Retrieved from the world wide web at alexionpharmaceuticals.com/news/sub press.cfm?prid=242&selectyear=2002.	
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	4	Alexion Pharmaceuticals Signs Agreement with Lonza Biologics for Commercial Manufacturing; [online]; Alexion Pharmaceuitcals Inc.; [Retrieved on 2003-06-17]; Retrieved from the world wide web at noonanrusso.com/news/view newsitem.aspx?ItemID=394.	

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/148,839	05/06/2016	Leonard BELL	AXJ-114BUSCN2

CONFIRMATION NO. 3159

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Nelson Mullins Riley & Scarborough LLP/Alexion
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30th Floor
Boston, MA 02109-2127

PUBLICATION NOTICE

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

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The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/148,839	05/06/2016	Leonard BELL	AXJ-114BUSCN2	3159

133485 7590 09/14/2016

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
09/14/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

Applicant-Initiated Interview Summary	**Application No.** 15/148,839	**Applicant(s)** BELL ET AL.	
	Examiner JAMES ROGERS	**Art Unit** 1644	

All participants (applicant, applicant's representative, PTO personnel):

(1) *JAMES ROGERS*.

(2) *SHARON WEN*.

(3) *JANE REMILLARD*.

(4) *DARRELL FONTENOT*.

Date of Interview: *07 September 2016*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1*.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Proposed amendments to the outstanding rejection of record was discussed. No agreement was reached.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/SHARON WEN/ Primary Examiner, Art Unit 1644	/James Rogers/ Examiner, Art Unit 1644

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Samsung Bioepis Exhibit No. 1002
Page 9795

PTO/AIA/25 (04-13)
Approved for use through 04/30/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) AXJ-114BUSCN2

In re Application of: Leonard BELL *et al.*

Application No.: 15/148,839-Conf. #3159

Filed: May 6, 2016

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

The applicant, Alexion Pharmaceuticals, Inc., owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number (see attached), filed, _____, as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said **reference** application, "as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application," in the event that: any such patent granted on the pending **reference** application expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. [X] The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. [ ] The undersigned is an attorney or agent of record. Reg. No. _____



Signature

02/14/17

Date

Todd Spalding

Typed or printed name

Vice President, Head of Intellectual Property

Title

_____

Telephone Number

[X] Terminal disclaimer fee under 37 CFR 1.20(d) is included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Samsung Bioepis Exhibit No. 1002
Page 9796

SUPPLEMENTAL SHEET FOR TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) AXJ-114BUSCN2

Reference application Nos. applicable to this terminal disclaimer (referenced on first page):

15/260,888 (filed September 9, 2016)

15/284,015 (filed October 3, 2016)

Samsung Bioepis Exhibit No. 1002
Page 9797

Corrected Application Data Sheet

Inventor Information

Inventor Number::	1
Given Name::	Leonard
Family Name::	BELL
City of Residence::	Woodbridge
State or Province of Residence::	CT
Country of Residence::	US
Street of mailing address::	59 Tumblebrook Road
City of mailing address::	Woodbridge
State or Province of mailing address::	CT
Country of mailing address::	US
Postal or Zip Code of mailing address::	06525

Inventor Number::	2
Given Name::	Russell
Middle Name::	P.
Family Name::	ROTHER
City of Residence::	Oklahoma City
State or Province of Residence::	OK
Country of Residence::	US
Street of mailing address::	10325 Forest Meadow Drive
City of mailing address::	Oklahoma City
State or Province of mailing address::	OK
Country of mailing address::	US

Postal or Zip Code of mailing address::	73151

Correspondence Information

Correspondence Customer Number::	133485
E-Mail address::	ipboston.docketing@nelsonmullins.com

Application Information

Application Number::	15/148,839
Filing Date::	05/06/16
Title::	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Attorney Docket Number::	AXJ-114BUSCN2

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::	Prior Appl Status::
This Application	Continuation of	13/426,973	03/22/12	Pending
13/426,973	Continuation of	12/225,040	05/13/09	Pending
12/225,040	National Stage of	PCT/US2007/006606	03/15/07	
~~PCT/US2007/006606~~	~~An application claiming the benefit under 35 USC 119(e)~~	~~60/783070~~	~~03/15/06~~	

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2017-02-15
Name	Jill Gorny Sloper, Esq.	Registration Number	60,760

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15148839 Document Date: 02/15/2017

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

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Form Revision Date: August 26, 2013

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Page 9801

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: February 15, 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

REQUEST FOR CORRECTED FILING RECEIPT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant hereby requests that a corrected Filing Receipt be issued in the above-identified patent application to reflect an update to the Domestic Priority information.

Please correct the Domestic Priority data as claimed by applicant from:

This application is a CON of 13/426,973 03/22/2012
which is a CON of 12/225,040 05/13/2009 ABN
which is a 371 of PCT/US2007/006606 03/15/2007
which claims benefit of 60/783,070 03/15/2006

to:

This application is a CON of 13/426,973 03/22/2012
which is a CON of 12/225,040 05/13/2009 ABN
which is a 371 of PCT/US2007/006606 03/15/2007
~~**which claims benefit of 60/783,070 03/15/2006**~~

Samsung Bioepis Exhibit No. 1002
Page 9802

Applicant additionally requests that all pertinent U.S. Patent and Trademark Office records relating to the subject application be changed to reflect this correction.

Applicant believes no fee is due with this statement. However, if a fee is due, please charge our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2 from which the undersigned is authorized to draw.

Dated: February 15, 2017

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

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: February 15, 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

MS Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT PURSUANT TO 37 CFR 1.821

Dear Sir:

Submitted herewith in connection with the above-referenced patent application and in full compliance with 37 C.F.R. §§1.821-1.825 is a computer readable copy and paper copy of the Sequence Listing (filed together as a .txt file via the United States Patent Office's Electronic Filing System).

I hereby state that I have reviewed the paper copy of the Sequence Listing, as required by 37 CFR 1.821(c), and have reviewed the computer readable form of the Sequence Listing, as required by 37 CFR 1.821(e), and that the content of the paper and computer readable copies for the above-referenced Patent application are the same as required by 37 CFR 1.821(f) (note that these documents are submitted as one electronic file). No new matter has been added to the Sequence Listing.

Samsung Bioepis Exhibit No. 1002
Page 9804

Applicant believes no fee is due with this statement. However, if a fee is due, please charge our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2 from which the undersigned is authorized to draw.

Dated: February 15, 2017

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

REMARKS

Claim 1 was pending. Claim 1 has been amended and new claims 2-3 have been added. Support for the claim amendments can be found throughout the specification as originally filed and as discussed below (see, *e.g.*, page 21 (line 23) through page 26 (line 27) and page 44 (lines 24-29)).

The claim amendments presented herein should in no way be construed as an acquiescence to any of the Examiner's rejections and have been made solely to expedite prosecution of the application. Applicants reserve the right to pursue claims to any canceled subject matter, or any subject matter which they are entitled to claim, in this or a separate application. *No new matter has been added.*

Information Disclosure Statement

Applicants acknowledge with appreciation the Examiner's indication that the Information Disclosure Statements submitted on May 6, 2016 have been considered. Additionally, Applicants submit herewith a Supplemental Information Disclosure Statement to make of record additional information for the Examiner's consideration.

Priority Claim

Applicants submit herewith a corrected ADS to amend the priority claim, *i.e.*, to remove the priority claim to U.S. Provisional Application Serial No. 60/783,070 (filed 15-Mar-2006). In addition, Applicants have amended the specification to include a "Cross Reference to Related Applications" section.

Replacement Sequence Listing and Amendment to the Specification

It has come to Applicants' attention that the light chain recited in the specification and Sequence Listing as SEQ ID NO:4 inadvertently contains the precursor peptide leader sequence, as shown below:

SEQ ID NO: 4 (Light Chain) MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASE NIYGALNWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTIS

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SLQPEDFATYYCQNVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQL
KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Accordingly, Applicants submit herewith a replacement Sequence Listing amending SEQ ID NO:4 to delete the leader sequence (underlined above), which is ultimately cleaved from the active light chain. Thus, as amended, SEQ ID NO:4 corresponds to the mature portion of the complete light chain sequence of eculizumab. Applicants have also amended the specification to update the "Sequences" summary on page 44 (lines 24-29) of the specification.

Applicants respectfully note that the mature light chain sequence is an inherent portion of the precursor sequence (*i.e.*, original SEQ ID NO:4), and could have been readily identified at the relevant filing date using well established rules and art-recognized techniques (see, for example, Figure 6 on page 734 of Adderson *et al.*, *J. Clin. Invest.* 89:729-738 (1992) (enclosed herewith as Exhibit A), which shows the characteristic mature N-terminus (DIQ) of a light V kappa antibody light chain, as well as Kabat *et al.*, Sequences of Immunological Interest 5th Edition, U.S. Public Health Service, Bethesda MD). Therefore, the amendment to SEQ ID NO:4 does not constitute new matter.

Terminal Disclaimer

Applicants submit herewith a terminal disclaimer over U.S. Patent Application No. 15/260,888 and U.S. Patent Application No. 15/284,015.

Rejection Under 35 U.S.C. § 103

Claim 1 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Hillmen *et al.* (*N. Engl. J. Med.* 2004; 350; 552-9), in view of Evans *et al.* (US 6,355,245), Wang (2005/0271660), as is evidenced by the parent specification of US Patent Application No.: 13/426,973.

Applicants respectfully traverse the rejection. As amended, the pending claims are drawn to pharmaceutical compositions comprising eculizumab as defined by its particular heavy and light chain sequences, which were not taught or suggested by the prior art.

Specifically, 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; nor was it available to the public. Hillmen *et al.* fails to teach any part of the sequence of eculizumab. Evans *et al.* and/or Wang, either alone or in combination, fail to cure this deficiency. Importantly, Evans *et al.* fails to teach or in any way suggest the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab (including the CH1-hinge-CH2-CH3 regions). Moreover, Wang fails to teach any part of the sequence of eculizumab, let alone its unique heavy chain.

Accordingly, for at the least the above-discussed reasons, the pending claims are patentable and the Examiner is respectfully requested to reconsider and withdraw this rejection.

CONCLUSION

If a telephone conversation with Applicants' attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' attorney at (617) 217-4630. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2.

Dated: February 15, 2017

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

EXHIBIT A
Adderson *et al.*, *J. Clin. Invest.* 89:729-738 (1992)

Samsung Bioepis Exhibit No. 1002
Page 9810

Downloaded from http://www.jci.org on January 19, 2017. https://doi.org/10.1172/JCI115649

Immunoglobulin Light Chain Variable Region Gene Sequences for Human Antibodies to *Haemophilus influenzae* Type b Capsular Polysaccharide Are Dominated by a Limited Number of V_κ and V_λ Segments and VJ Combinations

Elisabeth E. Adderson,* Penelope G. Shackelford,* Richard A. Insel,§ Anthony Quinn,* Patricia M. Wilson,‡ and William L. Carroll‖

*Division of Infectious Diseases and ‡Hematology Oncology, Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis Children's Hospital, St. Louis, Missouri 63110; and §Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Abstract

The immune repertoire to *Haemophilus influenzae* type b capsular polysaccharide (Hib PS) appears to be dominated by certain light chain variable region genes (IgV_L). In order to examine the molecular basis underlying light chain bias, IgV_L genes have been cloned from a panel of heterohybridomas secreting human anti-Hib PS (antibody) (anti-Hib PS Ab). One hybridoma, representative of the predominant serum clonotype of anti-Hib PS Ab in older children and adults following immunization or Hib infection, uses a V_κII segment identical to the germline gene A2, and a JK3 segment. A second kappa hybridoma uses a member of the V_κI family and a JK4 segment. Four lambda antibodies, all cross-reactive with the structurally related antigen *Escherichia coli* K100 PS, use V_λVII segments which are 96–98% homologous to one another, and may originate from a single germline gene. Two additional lambda antibodies, not K100–cross-reactive, are encoded by members of the V_λII family. All lambda antibodies use highly homologous J_λ2 or J_λ3 segments. The VJ joints of all lambda antibodies and the V_κII–encoded antibody are notable for the presence of an arginine codon, suggesting an important role in antigen binding. Although more complex than heavy chain variable region gene usage, a significant portion of serum anti-Hib PS Ab is likely to be encoded by a limited number of V_κ and V_λ segments and VJ combinations, which may be selectively expressed during development, or following antigen exposure. (*J. Clin. Invest.* 1992: 89:729–738.) Key words: B-cell repertoire • somatic mutation • gene rearrangement • carbohydrate antigen

Introduction

The development of the antibody repertoire involves the recombination of individual members of two or three groups of germline segments, nucleotide insertion or deletion at these junctions, and somatic mutation of rearranged genes (1–4). The individual contributions of these molecular mechanisms to the generation of the immune repertoire against a specific antigen has been extensively studied in murine systems, but it is uncertain whether these findings can be extrapolated to humans. The chromosomal organization of the human heavy and light chain variable region segments within the immunoglobulin loci differ from those of mice (5–7). Furthermore, the response of highly inbred laboratory animal strains, and the use of simple haptens in most studies, may not be comparable with the human response to clinically relevant antigens, such as those presented by microbial pathogens.

The immune response to *Haemophilus influenzae* type b capsular polysaccharide (Hib PS)[1] is an excellent model to examine the immune repertoire to a functionally significant antigen. Hib is a major bacterial pathogen in young children and antibody directed against the capsular polysaccharide, a repetitive polymer of polyribosylribitol phosphate, protects against invasive infection (8). Similar to the response to other polysaccharide antigens, the immune response to Hib PS is age-dependent. Children under 5 yr have lower antibody responses and are at greatest risk of infection. Additionally, there appear to be variations in individual and ethnic susceptibilities to invasive Hib disease (9–11). Analysis of the immune response to Hib PS will contribute to our understanding of the development of the human antibody repertoire, and provide insight into age-related and possible genetic predisposition to infection caused by Hib.

Previous studies employing partial amino acid sequencing of anti-Hib PS serum antibody purified to clonality have shown that light chains of anti-Hib PS antibody are encoded by V_λ, and at least three V_κ gene families (12). Although highly informative, these studies have inherent technologic limitations. To obtain sufficient antibody for analysis, only individuals with antibody concentrations much higher than those elicited in young infants could be studied (12). Amino acid sequences were unobtainable on V_λ-encoded antibodies, and certain regions of V_κ-encoded antibodies (12, 13). Obtaining nucleic acid sequences of variable region genes encoding anti-Hib PS Abs would permit precise comparison of variable region genes obtained from different individuals, allowing classification of these genes into germline families and subfamilies, analysis of D- and J-segment usage, and assessment of the role of somatic mutation in the clonal evolution of this immune response. Additionally, this information could be applied to examination of the germline and expressed immune repertoire

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Address correspondence and reprint requests to Dr. William L. Carroll, Program in Human Molecular Biology and Genetics, University of Utah, Bldg. 533, Salt Lake City, UT 84112.

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1. *Abbreviations used in this paper:* Ars, anti-p-azophenylarsonate; Hib, Haemophilus influenzae type b; Hib PS, Hib capsular polysaccharide; IgV_L, light chain variable region gene; PCR, polymerase chain reaction; SSC, standard saline citrate.

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of individuals of various ages, and populations at high risk for invasive Hib infection.

Our previous work, and that of others, indicated that the heavy chains of anti-Hib PS Abs are encoded by a very restricted number of V_HIII gene segments, in combination with a variety of D and J_H segments (12, 14). We have extended our studies to examine light chain variable region gene (IgV_L) usage in the human anti-Hib PS response. A major component of serum anti-Hib PS Ab expresses a cross-reactive idiotype associated with the use of a V_κII gene (15, 16). Antibodies expressing this light chain account for the majority of anti-Hib PS antibody in older infants and adults following invasive Hib disease or immunization. Most lambda-encoded anti-Hib antibodies and some kappa-encoded (V_κI and V_κIII) anti-Hib PS Abs cross-react with the capsular polysaccharide of *E. coli* K100 (12, 17). Naturally acquired antibody of the majority of children expresses K100 PS cross-reactivity, and thus, colonization with this organism may provide protective immunity before exposure to Hib (18). Our results indicate that two important anti-Hib PS light chains, one associated with V_κII expression and the other with lambda antibodies cross-reactive with K100 PS, are encoded by two germline elements that may be expressed with little or no somatic mutation. Moreover, a nongermline-encoded arginine at the VJ joint is seen in the majority of anti-Hib PS Ab light chains, a result suggesting an essential role in antigen recognition.

Methods

Hybridomas. Heterohybridomas secreting human monoclonal anti-Hib PS antibody were obtained, as previously described (14). Volunteers were immunized with either plain Hib-PS vaccine (Praxis Biologicals, Rochester, NY) or Hib-PS diphtheria toxoid conjugate vaccine (Hib-PS-D) (Connaught Laboratories, Swiftwater, PA). 7 d following immunization, peripheral blood lymphocytes were harvested and fused to the nonsecreting mouse myeloma SP2/O-Ag14. Anti-Hib PS Ab was detected by binding to Hib PS in an ELISA, as previously described (14). Specificity was documented by antibody binding to ^{125}I-labeled antigen (19), and by inhibition of binding by 1.25 μg/ml of soluble Hib PS in an ELISA (20). Cross-reactivity with *E. coli* K100 PS was assayed by inhibition of binding to Hib PS-poly-L-lysine with 100 μg/ml of soluble K100 PS in an ELISA. An antibody concentration was selected that gave half-maximum absorbance in the ELISA. Greater than 15% inhibition was considered positive. The *E. coli* K100 PS was prepared from *E. coli* strain Easter, by the same method used to prepare Hib PS (19). The generation of hybridoma line 16M3C8 was described previously (21).

Cloning and sequencing of IgV_L genes. Total RNA was prepared from 10^6 hybridoma cells using the guanidine isothiocyanate method (22). 2 to 8 μg of total RNA served as template for first-strand cDNA synthesis, as previously described (14). 10 pmol of a consensus antisense primer corresponding to conserved sequences within the human kappa or lambda constant region (23) (C_κ codons 181-172:5'CAGCGTCAAGCTTCTGCTGTGGCTGTAGGT 3', C_λ codons 171-181:5'CAGGCTCAGGAAGCTTCTGGCCGCGTACTTGTT 3') was used to initiate cDNA synthesis by avian myeloblastosis virus (AMV) reverse transcriptase (RT; Life Sciences, St. Petersburg, FL). One half (10 μl) of the cDNA reaction was directly diluted into polymerase chain reactions (PCR), (24).

PCR conditions were as previously described, except that 70 pmol of an internal constant region consensus primer (C_κ codons 125-117:5'CAACGGATCCTCTGATGGCGGGAAGAT 3', C_λ codons 125-117:5'TGGGGATCCAGCTCCTCAGAGGAGGG 3') and 70 pmol of a degenerate 3' kappa or lambda leader sequence primer (kappa:5'GGGAATTCATGGACATG(GA)(GA)(GA)(GAT)(TC)CC-(ACT)(GAC)G(TC)(GT)CA(GC)CTT 3', lambda 5'GGGAATTCATG(GA)CCTG(GC)(AT)C(TC)CCTCTC(TC)T(TC)CT(GC)(AT)-(TC)C 3') were used to amplify rearranged variable region genes (14, 25).

PCR products were isolated from low melting-point agarose gels (FMC Bioproducts, Rockland, ME). Artificial restriction enzymes sites in each primer allowed directional cloning into M13mp18 and M13mp19 phage vectors (Boehringer Mannheim Corp., Indianapolis, IN). Sequencing of V_L genes was performed by the dideoxy technique (26). Two to five positive clones were obtained and sequenced for each hybridoma gene.

The 16M3C8 light chain was generated by amplification of first-strand cDNA, with primers corresponding to amino acid positions −7 of the leader, to position 6 of the lambda light chain (5'CTGCACAGGGTCCTGGGCCGAGCTCGTGGTGACTCA 3'), and the C_λ constant region (antisense, 5'GCATTCTAGACTATTATGAACATTCTGTAGGGGC3'). Blunt-ended PCR products were cloned into SmaI cut M13 mp18 and M13mp19 and sequencing was also performed by the dideoxy method.

Southern blot analysis of genomic DNA. Peripheral blood was obtained from 10 unrelated healthy adult volunteers, from L.S. (the donor of hybridoma LSF2), and from a parent of L.S. Lymphocytes were separated on a Ficoll-Hypaque gradient, and genomic DNA was prepared by Proteinase K digestion (27). 10 μg of each subject's genomic DNA was digested with BamHI (4 U/μg DNA) (Boehringer Mannheim Corp.) at 37°C for 16 h, according to manufacturer's specifications. Digested DNA was separated by electrophoresis in a 0.8% agarose gel using TAE buffer (0.04 M Tris-acetate/0.001 M EDTA), and was transferred to Duralon UV membranes (Stratagene Inc., La Jolla, CA), using a positive pressure blotting apparatus (Posiblotter; Stratagene, Inc.). To examine germline representation of anti-Hib PS Ig genes, genomic DNA was hybridized with a 320-bp EcoRI/SmaI V_λ segment from hybridoma LSF2 that lacked the associated J_λ and C_λ regions. Probe was radiolabeled to high specific activity using T7 DNA polymerase (28). Prehybridization and hybridizations were carried out in 5× standard saline citrate (SSC), 5× Denhardt's solution, and 200 μg/ml sheared and denatured salmon sperm DNA (Boehringer Mannheim Corp.) at 42°C overnight. Filters were washed with 2× SSC, 0.2% SDS at room temperature twice, then with 0.2× SSC, 0.2% SDS at 55 to 60°C twice, and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for 2–7 d.

In order to further localize germline candidates within V region families, an oligonucleotide probe corresponding to conserved sequences within the CDR-1 region (sense oligonucleotide: 5'GGCTCCAGCACTGGAGATGTCACCAGT 3', antisense oligonucleotide: 5'GTAGGGATAATGACCACTGGTGACATCTCC 3') was hybridized to genomic DNA using the method described by Hellman (29). 15 μg of BamHI-digested human genomic DNA was separated by electrophoresis in 0.8% agarose gel using TBE buffer (0.09 M Tris-borate/0.002 M EDTA). Agarose gels were dried under vacuum and hybridized in 5× saline/sodium phosphate/EDTA (SSPE), 0.1% SDS, and 10^6 μg/ml of sheared and denatured salmon sperm DNA with 2×10^6 cpm of probe/ml of hybridization solution at 60°C for 16 h (30, 31). Hybridization conditions were previously adjusted to minimize nonspecific binding. Gels were washed with 6× SSC at room temperature for 20 min twice, followed by a 4-h room temperature wash. The gel was finally washed in 6× SSC for 3 min at 64°C, and exposed to X-OMAT AR film for 4 d.

Results

Hybridomas

Eight hybridoma cell lines from seven individuals were initially selected from 20 cell lines. All individuals demonstrated substantial increases in serum anti-Hib PS Ab 1 mo after vaccination (Table I). Hybridomas were selected to exemplify serologic characteristics (kappa or lambda expression, and K100 PS

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*Table I. Summary of Hybridoma Cell Lines**

Hybridoma	Isotype	Age	Vaccine Form	K100 Inh of mAb	Serum Anti-Hib PS Ab			
					Total Ab		ΔK100 Inh	
					Pre	Post	Pre	Post
RC3	IgA/k	Adult	PS-D	−	1.1	300	11	11
ED6.1	IgG_2/k	4 yr	PS-D	−	0.8	22	0	0
SB5/D6	IgA_1/λ	Adult	PS-D	+	2.0	688	30	27
RAY4	IgA_2/λ	Adult	PS-D	+	1.9	28	0	42
LSF2	IgA_1/λ	Adult	PS	+	ND	138	ND	29
JB32	IgA_1/λ	11 yr	PS	+	0.3	125	19	33
JB21	IgG_2/λ	11 yr	PS	−	0.3	125	19	33
16M3C8	IgG_2/λ	Adult‡	PS	−	ND	ND	ND	ND

* Columns show name of hybridoma cell line, isotype of anti-Hib PS Ab secreted, age of subject at immunization, type of vaccine (PS is plain polysaccharide, PS-D is Hib PS covalently linked to diphtheria toxoid), presence (+) or absence (−) of inhibition of binding of anti-Hib PS Ab by *E. coli* K100 PS, concentration of total serum anti-Hib PS AB pre- and 1 mo post-immunization, and percent inhibition of binding of serum lambda antibodies to Hib PS by *E. coli* K100 PS in pre- and 1 mo post-immunization sera. ‡ Pool of 3 adult donors. ND, not done.

cross-reactivity) and to represent the response to "T-independent" plain polysaccharide, or "T-dependent" polysaccharide-protein conjugate forms of antigen. (Table I).

Four lambda-expressing anti-Hib PS Ab, obtained from four different subjects, cross-reacted with K100 PS. One of these individuals, RAY4, had no detectable serum cross-reactivity before immunization. The Abs JB32 and JB21 were obtained from the same individual, and differed in heavy chain isotype and K100 cross-reactivity.

IgV_L genes. IgV_L nucleic acid sequences were obtained from two kappa and six lambda hybridomas. Candidate germline IgV_L sequences were sought by comparing hybridoma sequences to those registered with EMBL and GenBank (32).

Four lambda-expressing hybridomas, derived from four unrelated subjects, use V_λ segments which are 96–98% homologous to one another, and are members of the germline V_λVII family of which a single member, 4A, has been previously described (33) (Fig. 1). Notably, each of these four V_λVII-encoded hybridomas cross-reacts with K100 PS. The high degree of homology between these segments suggests that these genes may be encoded by a single, or group of, closely related germline elements. The four hybridoma genes share a number of nucleic acid differences (maximum nucleic acid sequence homology 89%) from the only previously sequenced V_λVII germline gene 4A, implying that these genes may be derived from another, as yet undescribed, V_λVII germline element.

The homology of the translated amino acid sequences of these 4 V_λVII sequences parallels the nucleic acid homology. The hybridoma V_λVII genes are 90 to 97% homologous at the amino acid level (Fig. 2). Framework regions are 94 to 100% homologous, whereas CDR regions are 72 to 96% homologous. Whether these minor differences reflect genetic polymorphisms, or somatic mutation, cannot be resolved from these data.

JB21 and 16M3C8, the lambda-expressing hybridomas which do not cross-react with K100 PS, are encoded by a V_λII gene family member (34), (Fig. 3). JB21 is 91% homologous to the germline V_λ2.1 gene (99% homologous in framework regions and 74% homologous in CDRs). 16M3C8 is 89% homologous to V_λ2.1 (95% in framework regions and 77% in CDRs). JB21 shares 87% amino acid homology with the translated V_λ2.1 germline sequence (99% in framework regions and 60% in CDR regions), and 16M3C8 shares 82% amino acid homology (92% in framework regions and 60% in CDRs) (Fig. 4).

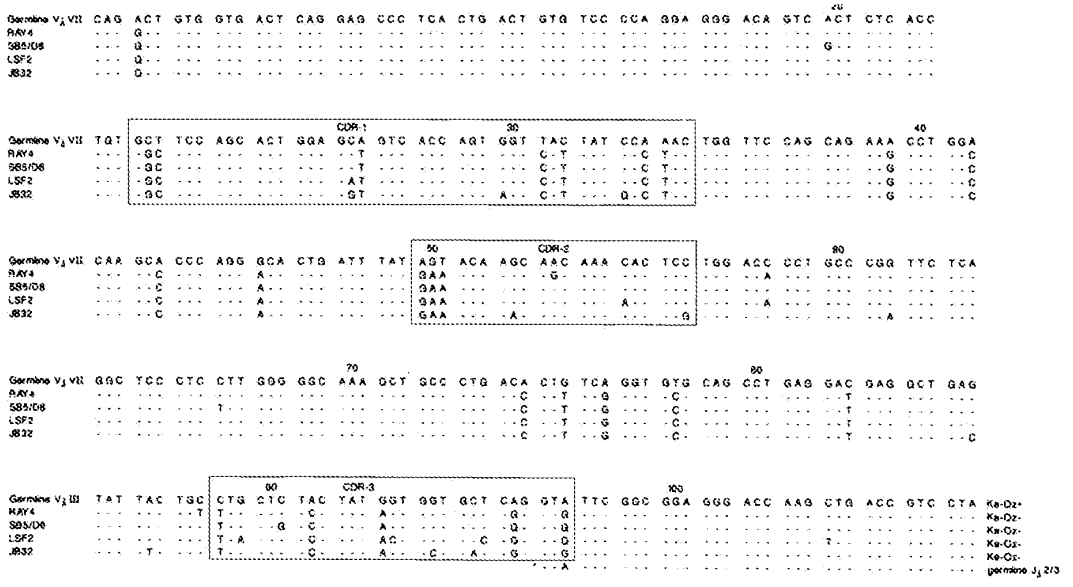


Figure 1. Nucleotide sequences of the V_λ and J_λ genes from four hybridoma cell lines (RAY4, SB5/D6, LSF2, JB32), and from the V_λVII germline gene, 4A (33). Numbering is according to Kabat (23). These sequence data are available from EMBL/GenBank Data Libraries under accession numbers M80917 (RAY4), M80918 (SB5/D6), M80915 (LSF2), and M80919 (JB32).

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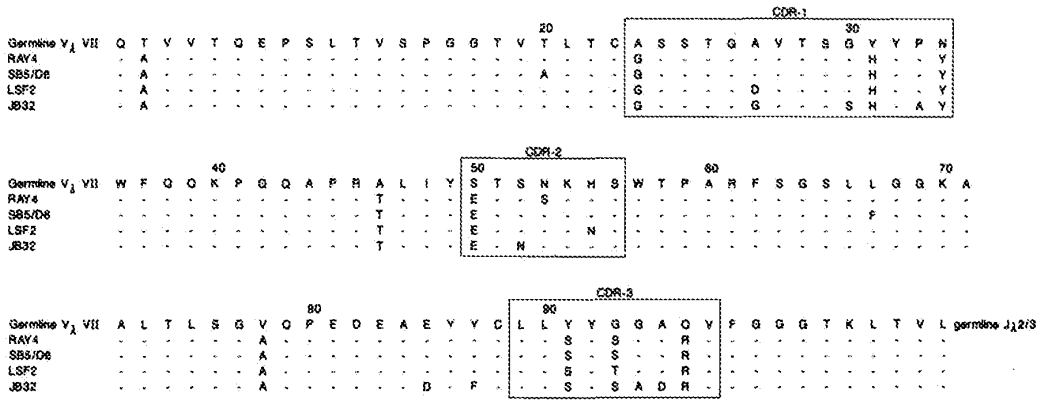


Figure 2. Translated amino acid sequences of V_λ and J_λ from four hybridoma cell lines (RAY4, SB5/D6, LSF2, JB32), and from the V_λVII germline gene, 4A (33). (See Fig. 1.)

These two V_λII-encoded gene segments are 91% homologous to one another, and have in common a number of nucleic acid differences from the V_λ2.1 germline gene (especially in CDRs). Whether they derive from another germline gene, or whether these differences reflect mutation, is unknown.

All four of the V_λVII-encoded antibodies are also encoded by V_HIII segments closely homologous to the germline gene 9.1 (5). Similar V_H segments also encode Ab utilizing other V_λ and V_κ gene segments (Table II) (manuscript in preparation). It is uncertain whether pairing of 9.1-like V_H and V_λVII-encoded heavy and light chains contributes to binding specificity or avidity. JB21 and RC3, also encoded by 9.1-like V_H gene segments, but paired with V_λII- and V_κII-encoded light chains, respectively, do not cross-react with K100 PS.

All six of the lambda light chains are encoded by a common J_λ segment closely homologous to both J_λ2 and J_λ3 germline genes (Fig. 1) (23). J_λ2 is associated with the use of the (Kern$^-$Oz$^-$)C_λ segment 2, and J_λ3 with the (Kern$^-$Oz$^+$)Cλ segment 3 (37). Each of these J_λ segments substitutes the codon GTG for the reported germline sequence GTA at amino acid position 97. Interestingly, this change does not result in an amino acid replacement, and is therefore not of importance in antigen binding. This minor sequence difference may reflect a shared genetic polymorphism, rather than somatic mutation.

The hybridoma RC3 uses a V_κII segment identical to the germline V_κII gene A2, and a J_κ3 segment closely homologous to the germline sequence (Fig. 5) (13). The second kappa hybridoma, ED6.1, is encoded by a member of the V_κI family

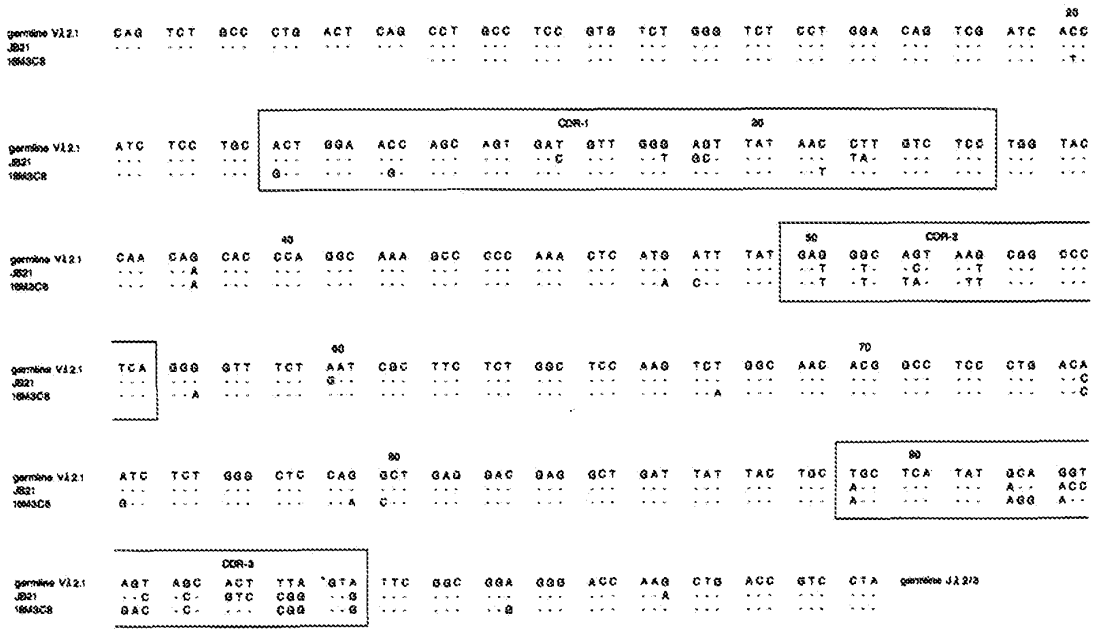


Figure 3. Nucleotide sequences of V_λ and J_λ genes from two hybridoma cell lines (JB21 and 16M3C8). Also shown is a germline gene V2.1 (34), and germline J_λ2/3 gene (23). An asterisk denotes the beginning of the J_λ segment. Numbering is according to Kabat (23). These sequence data are available from EMBL/GenBank Data Libraries under accession numbers M80916 (JB21) and M80921 (16M3C8).

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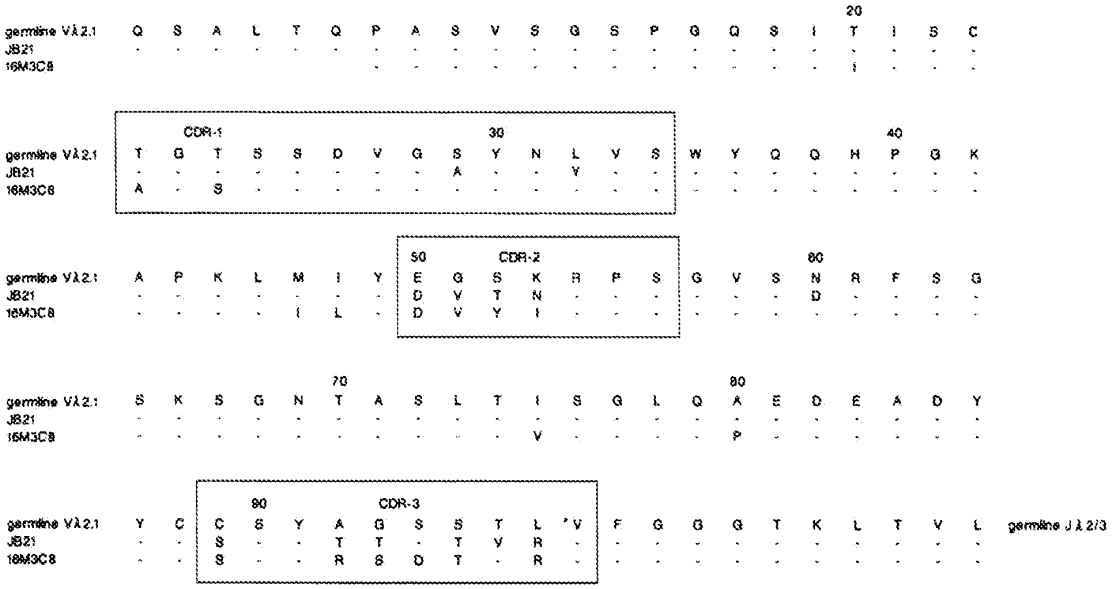


Figure 4. Translated amino acid sequences of V_λ and J_λ genes from two hybridoma cell lines (JB21 and 16M3C8). Shown for comparison is the translated sequence of the V_λ germline gene V_λ2.1, and J_λ2/3 germline gene. An asterisk denotes the beginning of the J_λ segment. (See Fig. 3.)

(36), and uses a J_κ4 segment differing from the germline sequence by a single base (Fig. 6). The ED6.1 sequence is identical to the partial amino acid sequence reported previously for 2 kappa antibodies purified from serum (12).

VJ joints of seven of these eight hybridoma V_L genes are notable for the presence of an arginine codon (Fig. 7). Comparison of germline sequences indicates that in the RC3 V_L gene, the codon CGA could only have arisen by the addition of a non–germline-encoded G at the $V_\kappa J_\kappa$ joint. In the V_λVII-encoded antibodies, the relevant germline V_λ segment is unknown. The arginine codon may be germline-encoded, or result from either mutation or N sequence addition. The VJ splice junction in all lambda antibodies is identical, implying this V_λ/J_λ combination is a necessary prerequisite for generation of the arginine. Since the CDR-3 region encodes the third hypervariable region, the frequent finding of an arginine residue at this location, and particularly its presence in both kappa and lambda anti–Hib PS Ab clonotypes, implies this residue is of considerable importance in antigen binding.

*Table II. Summary of V_H and V_L Gene Segments**

Hybridoma	V_H Segment	V_L Segment
SB5/D6	V_HIII 9.1 (5)	V_λVII 4A (33)
RAY4	V_HIII 9.1	V_λVII 4A
JB32	V_HIII 9.1	V_λVII 4A
JB21	V_HIII 9.1	V_λII 2.1 (34)
RC3	V_HIII 9.1	V_κII A2 (13)
LSF2	V_HIII 9.1	V_λVII 4A
16M3C8	V_HIII VH26 (35)	V_λII 2.1
ED6.1	N.K.	V_κI clone KC-1 (36)

* Columns show name of hybridoma cell line and most closely homologous known germline V_H and V_L segments. Reference for germline shown in parentheses. NK, Not known.

Southern blots/analysis of germline V_λVII genes

The available repertoire of V_λVII family members was examined by hybridization of human genomic DNA with the V_λVII probe generated from LSF2. 10 prominent bands, as well as an additional 5 fainter bands, were demonstrated (Fig. 8). Notably, polymorphism such as the presence or absence of a 5.0-kb fragment, and varying intensity of the 3.5-kb fragment, was demonstrated even within this relatively small sample. The human lambda variable region locus is believed to contain about 60 members, of which 10 are members of the V_λVII family (33, 38). The five less prominent bands may represent members of other V_λ families sharing significant homology with the V_λVII family. To determine if the V_λVII-encoded antibodies derive from a single germline gene, an oligonucleotide probe corresponding to conserved V_λVII CDR-1 sequence was used to identify a single 3.7-kb genomic DNA fragment. Although more than one V gene may be present in a single restriction fragment, both the repeated isolation of highly homologous V_λVII genes, and the single prominant band detected with the oligonucleotide probe, suggests that a single V_λ gene may be responsible for the generation of lambda anti–Hib PS Ab that cross-reacts with K100 PS (Fig. 9). Less prominant higher molecular weight bands are also present, but hybridize weakly to the oligomer after subtraction of nonspecific background binding by high molecular weight DNA.

Discussion

To examine the molecular basis of the immune response to Hib PS, a panel of hybridomas secreting human monoclonal anti–Hib PS Ab has been developed. Our previous studies showed exclusive use of members of the V_HIII gene family in this im-

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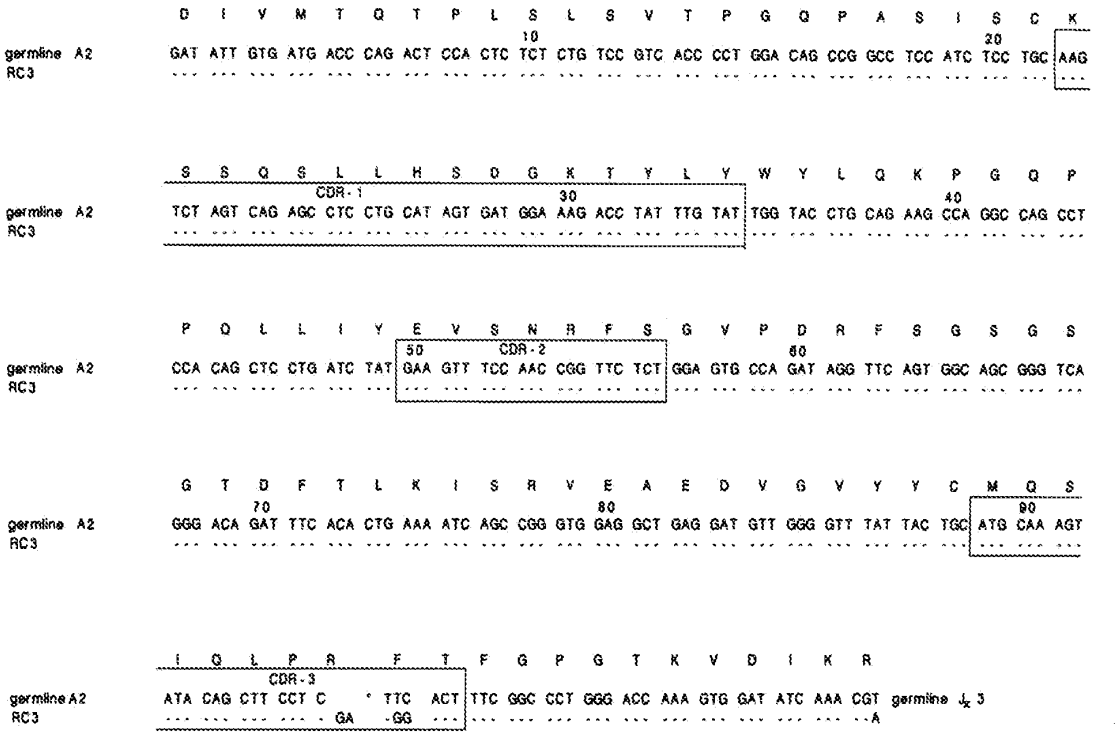


Figure 5. Nucleotide sequences of V_κ and J_κ genes from the hybridoma cell line RC3. Also shown is the translated amino acid sequence of the RC3 V_κ gene. Shown for comparison is the nucleotide sequence of a germline V_κ gene, A2 (13), and germline $J_\kappa 3$ gene. An asterisk denotes the beginning of the J_κ segment. Numbering is according to Kabat (23). This sequence data is available from EMBL/GenBank Data Libraries under accession number M80914 (RC3).

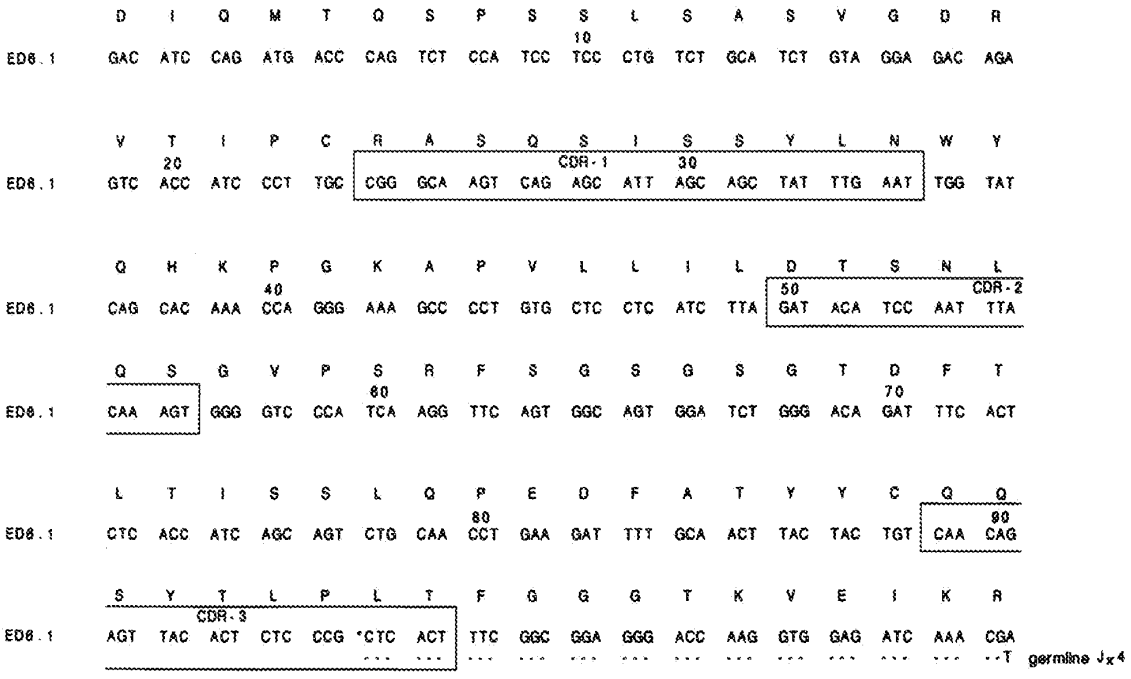


Figure 6. Nucleotide sequence of V_κ and J_κ genes from hybridoma cell line ED6.1. Also shown is the translated amino acid sequence of the ED6.1 V_κ gene. An asterisk denotes the beginning of the J_κ segment. Numbering is according to Kabat (23). This sequence data is available from EMBL/GenBank Data Libraries under accession number M80920 (ED6.1).

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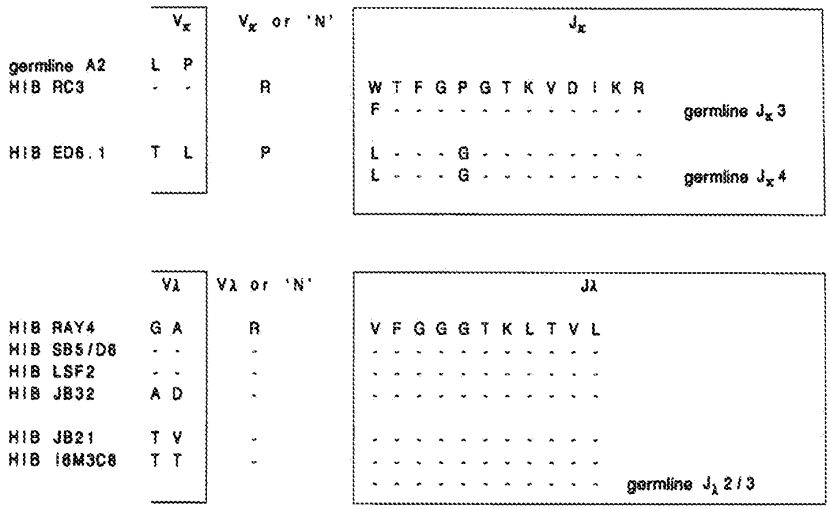


Figure 7. Translated amino acid sequences of VJ joints from seven hybridoma cell lines (RC3, ED6.1, RAY4, SB5/D6, LSF2, JB21, and 16M3C8). Also shown are translated amino acid sequences of the germline V_κ gene A2, and germline $J_\kappa 3$, $J_\kappa 4$, and $J_\lambda 2/3$ genes. (See Figs. 2, 4, and 6.)

mune response, and the apparent restriction to two or three germline V_H genes. In contrast, V_L gene usage is more complex, with antibody encoded by a number of V_λ and V_κ families (12). Analysis of light chain variable region genes obtained from cell lines selected to represent various characteristics of serum antibodies showed that despite diversity in the variety of light chain variable region gene segments utilized, most serum anti-Hib PS Abs may be encoded by a limited number of V_κ and V_λ germline genes.

The light chain distribution of anti-Hib PS Ab is characterized by a predominance of kappa light chains, although some individuals predominantly or exclusively express lambda light chains (39, 40). Scott et al. have shown that V_κII light chains are the most commonly expressed light chains among clonally purified serum anti-Hib PS antibodies from adults with high responses to immunization. Limited amino acid sequences of a number of these serum antibodies showed that greater than 60% of subjects immunized with plain Hib PS produce antibody encoded by a V_κII family member, and further, that a number of these antibodies show identical amino acid homology with the translated amino acid sequence of the germline V_κII gene A2 (12, 13). Lucas et al. have described an anti-idiotypic monoclonal antibody (Hibid-1) that identifies anti-Hib PS antibodies expressing this light chain (16). On average, 60%

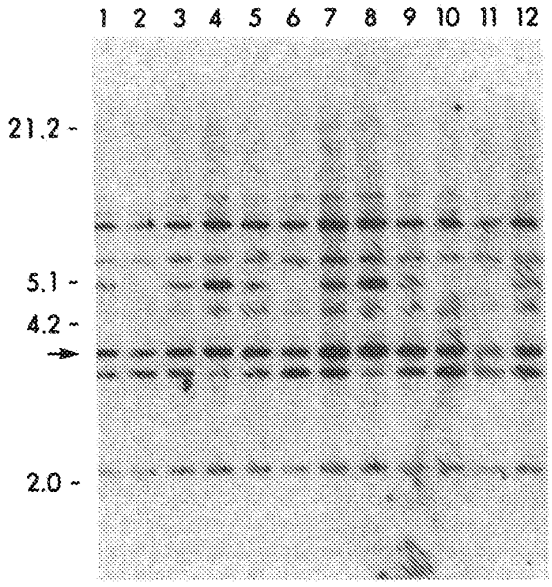


Figure 8. Southern blot analysis for V_λVII family members in BamHI-digested human genomic DNA. Lane *12* contains genomic DNA from the subject from whom hybridoma LSF2 was obtained. Lane *9* contains genomic DNA from a parent of LS. The remaining 10 donors are unrelated. An arrow indicates the 3.7-kb fragment which is identified by the oligonucleotide probe illustrated in Fig. 9.

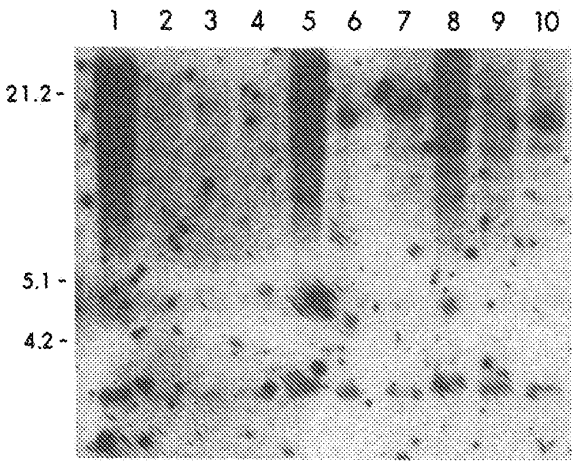


Figure 9. Dried gel hybridization of BamHI-digested human genomic DNA with an oligonucleotide probe corresponding to conserved V_λVII hybridoma CDR-1 sequences. Genomic DNA in lanes *1, 4, 5, 6, 7, 9* and *10* of Fig. 8 corresponds to that in lanes *2, 7, 4, 8, 10, 5* and *9* of Fig. 9, respectively.

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of the total anti-Hib PS antibody of most children and adults immunized with either Hib PS or Hib PS-conjugate vacine expresses this idiotype (15, 16). Taken together, this suggests the most common clonotype of anti-Hib PS Ab is encoded by a single V_κII gene. The V_κ locus contains approximately 50 functional genes, of which 40% are members of the V_κII family (41). The V_κII A2 germline gene is known to be present in a single copy (13). Our findings definitively demonstrate that at least a portion of V_κII-encoded antibodies are encoded by unmutated germline elements. Our subject R.C. was immunized with PS-protein conjugate vaccine, whereas Scott's subjects were immunized with the plain PS vaccine. Thus it is possible that the unmutated V_κII A2 segment is utilized irrespective of antigen presentation in a T-independent or T-dependent form.

Lambda variable region gene usage appears similarly restricted. The majority of lambda-expressing anti-Hib PS Ab in adults with high antibody responses to vaccine cross-reacts with *E. coli* K100 PS (17). The capsular polysaccharides of Hib and *E. coli* K100 are structurally similar and gastrointestinal colonization with *E. coli* K100 induces antibody to Hib PS in humans (42). "Naturally occurring" anti-Hib PS Ab in children commonly cross-reacts with K100 PS, whereas Ab of individuals immunized with Hib PS or responding to Hib infection is less frequently cross-reactive (18). The kappa/lambda ratio of natural anti-Hib PS Ab is lower in infants than in older children, and in the vaccine-induced antibody of adults (39). Further, Hibid-1 (the CRI associated with expression of the V_κII light chain) is expressed less frequently and comprises a smaller proportion of total anti-Hib PS Ab in preimmune compared with postimmunization sera. Expression of Hibid-1 following immunization is similar in adults and children. These results imply that cross-reactive antigens may induce the natural antibody of children. K100 PS-cross-reactive antibody may provide protective immunity in young children upon first exposure to Hib. These cross-reactive antibodies appear to be subsequently replaced or dominated by antibody specific for Hib PS when individuals are exposed to this antigen by immunization, colonization, or infection. We demonstrate that cross reactivity of lambda antibodies may be mediated through a single light chain germline gene. The mechanism for the clonal dominance of the V_κII antibodies is unclear; the avidity for Hib PS of these K100 PS–cross-reactive antibodies from adults is similar to that of Hib specific PS antibodies (P. Shackelford, unpublished observation).

Lambda antibodies expressing the V_λII gene did not cross-react with *E. coli* K100 PS. Whether all noncross-reactive antibodies are encoded by closely related members of the V_λII gene family will require further characterization of additional antibodies.

By the age of about 5 yr most individuals have acquired protective anti-Hib PS Ab. All of the hybridomas in this study were derived from older children or adults, who had detectable preimmunization antibody. The B cells immortalized by fusion, therefore, most likely represent a secondary immune response. Despite this, V_H gene segments obtained from these hybridomas appear to be relatively unmutated (14), and it appears that some V_L genes are similarly conserved. The RC3 V_κ gene segment is identical to the A2 germline gene, and although the pertinent V_λVII germline gene is not known, the paucity of differences in V_λVII segments from four different subjects suggests these gene segments are relatively unmutated. J_κ and J_λ segments are also highly homologous to germline elements. Thus, at least a portion of the anti-Hib PS response represents the use of unmutated germline elements.

VJ joints of the majority of rearranged V_L genes also appear to be an additional source of restriction in the anti-Hib PS immune response. Although there is variability in the use of J_κ segments, V_κII-encoded V_L genes are characterized by the presence of an arginine at amino acid position 95a (13). In the RC3 V_L gene and at least one of the antibodies (B-G2a) described by Scott, this appears to have arisen by N sequence addition. In four other kappa antibodies described by Scott, alternative joining may have generated this invariant arginine (13). All of the lambda V_L genes in the present study are similarly characterized by an invariant arginine-96. The germline V_λVII segment is not known; therefore the mechanism used to generate this codon is uncertain. The invariant presence of this residue in many anti-Hib PS Abs supports the previous suggestion that it may be of great importance in antigen binding (13). This also is supported by studies of the murine anti-*p*-azophenylarsonate (Ars) response. Anti-Ars light chains also contain an invariant arginine at position 96, encoded by intracodonic splicing of a single V_κ (V_κ10-Ars) and a single J_κ (J_κ1) segment (43). This arginine is essential for Ars binding, and the substitution of tyrosine for arginine has been shown to abolish binding (44). The presence of arginine residues clustered within the CDR's of the heavy chains of murine anti-DNA antibodies correlates with increased affinity, and the arginine requirement in the anti-Hib PS response may indicate its participation in the recognition of the similar phosphate–ester linkage of the repeating carbohydrate moiety (45). Thus, although diversity exists in the use of a variety of V and J segments, certain VJ combinations, which produce arginine codons by junctional flexibility or N segment addition, may be strongly selected.

Does the restricted nature of V_H and V_L segment usage in the human anti-Hib PS response explain the age-related acquisition of immunity to this organism? In both mice and humans, preferential use of certain V_H gene segments is observed in early development. In mice, biased usage of J_H-proximal V_H gene segments occurs in fetal and neonatal animals (46, 47). Developmental regulation of V_L gene usage is less extensively studied than V_H genes. Teale and Morris noted preferential expression of certain V_κ families in LPS-stimulated murine fetal B cells compared to adult B cells, with no evidence for position-dependent bias (48). Kaushik et al. also described nonstochastic use of V_κ families in adult mice, suggesting molecular constraints may persist beyond the neonatal period (49). Two or three V_H gene segments, and a single V_κ and a single V_λ gene segments, appear to encode a large proportion of anti-Hib PS Ab in immune subjects. The use of highly conserved V gene segments in human anti-Hib PS response may place considerable restraints on the immune response. The inability to utilize these genes, whether due to chromosomal position or other molecular constraints, may be responsible for the age-related susceptibility to Hib infection. The lack of somatic hypermutation in a portion of these antibodies would also place greater dependence on the germline repertoire, in that it may be impossible to improve antibody affinity by antigen-driven selection of favorable mutations. The absence or polymorphism of these genes also may contribute to individual and ethnic susceptibility to invasive Hib disease. In addition, the repertoire differences detected in some individuals (such as the absence of kappa antibody, or the absence of the V_κII-associated CRI) may be explained by the use of other minor light chains. An individ-

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ual missing the A2 germline gene has been described, although the functional implications of such a deletion are not known (13).

Expression of certain V_H gene family members in mice is correlated with B cell subpopulations (50). An alternative explanation for the poor response to plain Hib PS may be a maturational delay in recruitment of a critical B cell population that selectively expresses important variable region gene segments. We and others note that an arginine is expressed at the VJ joint in the great majority of anti-Hib PS light chains. In some circumstances N segment addition is required to generate this codon. The addition of these extra nucleotides is infrequent in light chain recombination, suggesting that these B cell precursors may be relatively infrequent (51). Moreover, N segment addition also appears to be developmentally regulated. Neonatal murine B cells lack N sequences, and their presence in heavy chain recombination increases with time (52). In addition, N sequence addition may also correlate with certain B cell subsets. N segments appear to be rare in Ly1-B cell-derived populations (53).

In contrast to plain polysaccharide vaccines, most young infants respond well to the newer Hib-PS protein conjugate vaccines (54). It is presently unknown if this antigen formulation recruits an alternative antibody repertoire, or if, by an uncertain mechanism, developmental restriction of a "dormant" B cell subset is overcome. To address this question, V gene expression in infants must be examined.

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AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

1. **(Currently Amended)** A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a sterile, preservative free, 300 mg ~~eculizumab~~ single-use dosage form comprising 30 ml of a 10 mg/ml antibody ~~eculizumab/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.

2. **(New)** A pharmaceutical composition comprising an anti-C5 antibody, wherein the anti-C5 antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

3. **(New)** The pharmaceutical composition of claim 2, wherein the pharmaceutical composition is a sterile, preservative free 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml anti-C5 antibody solution.

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AMENDMENTS TO THE SPECIFICATION

In the Sequence Listing:

Please replace the Sequence Listing filed on May 6, 2016 with the replacement Sequence Listing submitted herewith.

In the Specification:

Please insert the following paragraphs after the title:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application No. 13/426,973, filed March 22, 2012, which is a continuation of U.S. Patent Application No. 12/225,040, filed on May 13, 2009, which is a 35 U.S.C. 371 national stage filing of International Application No. PCT/US2007/006606, filed March 15, 2007. The contents of the aforementioned applications are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 13, 2017, is named AXJ_114BUSCN2_Seq_Listing.txt and is 10,244 bytes in size.

Please amend the paragraph beginning on page 44 (lines 24-29) as follows:

SEQ ID NO: 4 – Eculizumab Light Chain
~~MDMRVPAQLLGLLLLWLRGARC~~DIQMTQSPSSLSASVGDRVTITCGASENIYGAL
NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

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: February 15, 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

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (SIDS)

Dear Sir:

In compliance with 37 C.F.R. § 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached PTO/SB/08. In accordance with 37 C.F.R. § 1.98(a)(2)(i)-(iv), Applicant submits herewith copies of foreign patents and non-patent literature references, but has not included copies of U.S. patents and U.S. patent applications.

It is respectfully requested that the documents listed on the PTO/SB/08 be expressly considered by the Examiner during the prosecution of this application, and that the documents be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

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Applicant calls to the attention of the Examiner the following Applications and provides copies of Office Action cited therein. Applicant also provides a copy of an Office Action from an Application previously made of record:

Applications				
Examiner's Initials	*Serial No.*	*Filing Date*	*First Named Inventor*	*Docket No.*
	15/260,888	09-09-2016	Leonard BELL	AXJ-114BUSCN3
	15/284,015	10-03-2016	Leonard BELL	AXJ-114BUSCN4

Office Actions (copies enclosed)			
Examiner's Initials	Serial No.	Date Mailed from USPTO	Examiner
	15/260,888	12-22-2016	J. Rogers
	15/260,888	10-27-2016	J. Rogers
	15/284,015	11-01-2016	J. Rogers
	13/335,518	08-22-2016	P. Gambel

The Examiner is requested to review the file histories of these applications, including cited references, Office Actions, Responses, etc., and is asked to contact Applicant's Attorney if the Examiner would like the Applicant to supply copies of any or all of the information included in any of these applications. For any of these applications, if Applicant's Attorney is not contacted by the Examiner with such a request, then it will be concluded that the Examiner has reviewed or will review the file content of these applications.

Applicant respectfully requests that the Examiner initial the blank columns next to the cited Applications and Office Actions, to indicate that the information has been considered by the Examiner. Alternatively, Applicant requests that the Examiner insert the phrase, "All references considered except where lined through," on each page of the Information Disclosure Statement, along with the Examiner's initials.

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Please charge our Deposit Account No. 12-0080 in the amount of $180.00 covering the fee set forth in 37 C.F.R. § 1.17(p). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN2.

Dated: February 15, 2017

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 217-4699 (Fax)
Attorney/Agent For Applicant

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(Not for submission under 37 CFR 1.99)

Application Number	15148839
Filing Date	2016-05-06
First Named Inventor	Leonard BELL
Art Unit	1644
Examiner Name	J. L. Rogers
Attorney Docket Number	AXJ-114BUSCN2

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	Attorney Docket Number	AXJ-114BUSCN2

	6	20050226870	A1	2005-10-13	Wang et al.	
	7	20060057679	A1	2006-03-16	O'Keefe et al.	
	8	20060263349	A1	2006-11-23	McCutcheon et al.	
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	2	2005110481	WO	A2	2005-11-24	Alexion Pharmaceuticals, Inc		
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	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN2

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	1	Adis International Limited, "Eculizumab: 5G1.1, h5G1.1, Long-Acting Anti-C5 Monoclonal Antibody 5G1-1, Long-Acting Anti-C5 Monoclonal Antibody 5G1.1," Drugs in Research and Development, vol. 8 (1): 61-68 (2007).	
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	3	Alexion Pharma K.K. "Report on the Deliberation Results" for "Soliris for intravenous infusion 300 mg", Marh 5, 2010, pp. 1-105	
	4	Alexion Pharmaceuticals Inc., "Alexion completes enrollment in Ph II kidney disease trial," Market letter (Feb 25, 2002), 2 pages.	
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	Attorney Docket Number	AXJ-114BUSCN2

	11	Alexion Pharmaceuticals Inc., "Eculizumab Alexion clinical data (phase III) (PNH)," R & D Focus Drug News (Feb 6, 2006), 2 pages.	
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	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN2

	33	Osborne, R., "Alexion Offers Early, Mixedphase III Data for Heart Drug," Bioworld Today, vol. 14, Issue 151 (Aug 6, 2003), 3 pages.	
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	Art Unit	1644
	Examiner Name	J. L. Rogers
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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2017-02-15
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

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International Application Number:	
Confirmation Number:	3159
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
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Attorney Docket Number:	AXJ-114BUSCN2
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Application Type:	Utility under 35 USC 111(a)

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(71) Applicants *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US). **THE UNIVERSITY OF WESTERN ONTARIO** [CA/CA]; 1151 Richmond Street North, Stevenson-Lawson Building, Room 328, London, Ontario N6A 5B8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROTHER, Russell, P.** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US). **WANG, Hao** [CA/CA]; 480 Sandybrook Drive, London, Ontario N5X 4H2 (CA). **ZHONG, Zhen** [CA/CA]; 60 Nathaniel Court, London, Ontario N5X 2N5 (CA).

(74) Agents: **WU, Xuqiong** et al.; Fish & Neave IP Group of, Ropes & Gray LLP, One International Place, Boston, MA 02110-2624 (US).

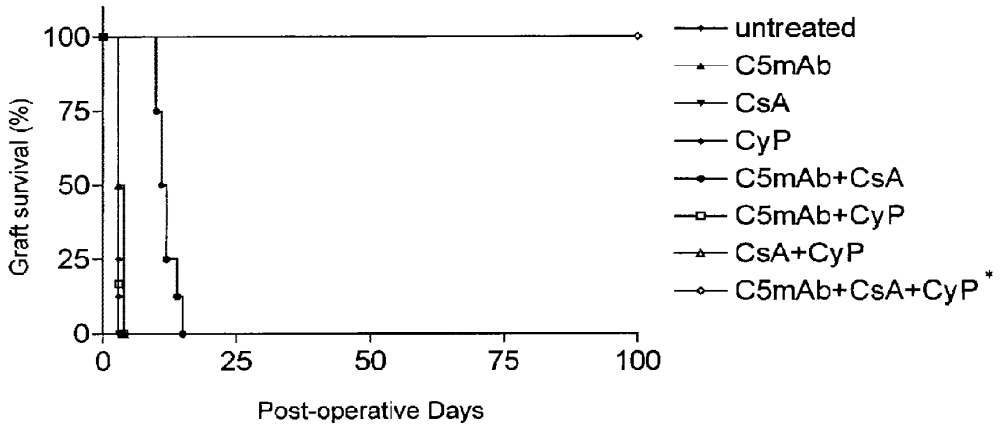
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(54) **Title:** PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY



(57) **Abstract:** Methods of prolonging survival of allotransplanted cells, tissues or organs are presented. These methods are directed to administering to the allotransplant recipient an inhibitor of complement activity together with one or more immunosuppressants. The inhibitor of complement activity is administered chronically. These methods have been determined to aid in preventing chronic rejection of allografts. These methods can additionally be used in cases in which the recipient has been presensitized to the allograft or in which there is an ABO mismatch between the allograft and the recipient.

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PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY

RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Patent Application No. 60/571,444, filed May 14, 2004, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to methods for prolonging survival of an allograft in a mammal. In particular, the present disclosure relates to prolonging survival of an allograft by administering an inhibitor of complement or terminal complement formation, especially an inhibitor of complement C5 cleavage, in addition to one or more drugs that are immunosuppressant.

BACKGROUND

Organ transplantation is the preferred treatment for most patients with chronic organ failure. Although transplantation of kidney, liver, lung, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle, it is limited by the medical/surgical suitability of potential recipients, an increasing shortage of donors, and premature failure of transplanted organ function.

Transplantation of cells, tissues and organs has become very common and is often a life-saving procedure. Organ transplantation is the preferred treatment for most patients with chronic organ failure. Despite great improvement in treatments to inhibit rejection, rejection continues to be the single largest impediment to successful organ transplantation. Rejection includes not only acute rejection but also chronic rejection. One-year survival rates for transplanted kidneys average 88.3% with kidneys from deceased donors and 94.4% with kidneys received from living donors. The corresponding five year survival rates for the transplanted kidneys are 63.3% and 76.5% (OPTN/SRTR Annual Report, 2002). For livers the one year survival rates are 80.2% and 76.5% for livers from deceased and living donors, respectively. The corresponding five year liver graft survival rates are 63.5% and 73.0% (OPTN/SRTR Annual Report, 2002). The use of immunosuppressant drugs, especially cyclosporin A and more recently tacrolimus, has dramatically improved the success rate of organ transplantation especially by preventing acute rejection. But as the numbers above

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show, there is still a need to improve the success rates, both short-term and especially long-term. As seen from the above numbers for kidney and liver transplants, the five year failure rates for these transplanted organs are on the order of 25-35%. In the year 2001 alone there were more than 23,000 patients who received an organ transplant of which approximately 19,000 were kidney or liver (OPTN/SRTR Annual Report, 2002). For this one year of transplants alone, with present techniques it can be expected that approximately 5,000-6,000 of these transplanted kidneys and livers will fail within 5 years. These numbers do not even include other transplanted organs or transplanted tissues or cells such as bone marrow.

There are multiple types of transplants. These are described in Abbas et al., 2000. A graft transplanted from one individual to the same individual is called an autologous graft or autograft. A graft transplanted between two genetically identical or syngeneic individual is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft or allograft. A graft transplanted between individuals of different species is called a xenogeneic graft or xenograft. The molecules that are recognized as foreign on allografts are called alloantigens and those on xenografts are called xenoantigens. The lymphocytes or antibodies that react with alloantigens or xenoantigens are described as being alloreactive or xenoreactive, respectively.

Currently more than 40,000 kidney, heart, lung, liver and pancreas transplants are performed in the United States each year (Abbas et al., 2000). Other possible transplants include, but are not limited to, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells. Unfortunately, there are many more candidates for a transplant than there are donors. To overcome this shortage, a major effort is being made to learn how to use xenografts. While progress is being made in this field, the fact is that at present most transplants are allografts. An allogeneic transplant, while presently being more likely to be successful than a xenogeneic transplant, must surmount numerous obstacles to be successful. There are several types of immunological attacks made by the recipient against the donor organ which can lead to rejection of the allograft. These include hyperacute rejection, acute vascular rejection (including accelerated humoral rejection and de novo acute humoral rejection), and chronic rejection. Rejection is normally a result of T-cell mediated or humoral antibody attack, but may include additional secondary factors such as the effects of complement and cytokines.

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An ever growing gap between the number of patients requiring organ transplantation and the number of donor organs available has become a major problem throughout the world. Park et al., 2003. Individuals who have developed anti-HLA antibodies are said to be immunized or sensitized. Gloor, 2005. HLA sensitization is the major barrier to optimal utilization of organs from living donors in clinical transplantation (Warren et al., 2004) due to the development of severe antibody-mediated rejection (ABMR). For example, more than 50% of all individuals awaiting kidney transplantation are presensitized patients (Glotz et al., 2002) who have elevated levels of broadly reactive alloantibodies, resulting from multiple transfusions, prior failed allografts, or pregnancy (Kupiec-Weglinski, 1996). The role of ABMR is currently one of the most dynamic areas of study in transplantation, due to recognition that this type of rejection can lead to either acute or chronic loss of allograft function. Mehra et al., 2003. Numerous cases of ABMR, including hyperacute rejection (HAR) or accelerated humoral rejection (ACHR), have been reported that are characterized by acute allograft injury that is resistant to potent anti-T cell therapy, the detection of circulating donor specific antibodies, and the deposition of complement components in the graft. ABMR with elevated circulating alloantibodies and complement activation that occurs in 20-30% of acute rejection cases has a poorer prognosis than cellular rejection. Mauiyyedi et al., 2002.

Highly presensitized patients, who exhibit high levels of alloantibodies, usually suffer an immediate and aggressive HAR. In clinical practice, with great efforts and significant advances in technology, HAR is avoided by obtaining a pretransplant lymphocytotoxic cross-match to identify sensitized patients with antibodies specific for donor HLA antigens. However, circulating antibodies against donor HLA or other non-MHC endothelial antigens may also be responsible for a delayed form of acute humoral rejection, which is associated with an increased incidence of graft loss. Collins et al., 1999. Therefore, development of a novel presensitized animal model to mimic ABMR in clinical settings would be beneficial to studies on the mechanism, and to the much needed progress in the management of allograft rejection in presensitized hosts.

Some highly presensitized patients can benefit from intervention programs such as immunoadsorption (Palmer et al.,1989; Ross et al., 1993; Kriaa et al., 1995), plasmapheresis and intravenous immunoglobulin (Sonnenday et al., 2002; Rocha et al., 2003), that have been designed and implemented to temporarily eliminate anti-donor antibodies. However, in addition to their benefits, the aforementioned therapies carry with them numerous drawbacks

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as some individuals are less susceptible to their effects (Kriaa et al., 1995; Hakim et al., 1990; Glotz et al., 1993; Tyan et al., 1994); they are extremely expensive, time-consuming, and risky (Salama et al., 2001). Moreover, the transient and variable effect of these protocols has limited their impact. Glotz et al., 2002; Kupin et al., 1991; Schweitzer et al., 2000. Therefore, developing novel strategies to reduce the risk and cost in prevention of ABMR would be beneficial to presensitized recipients receiving an allograft.

SUMMARY

Accordingly, methods of prolonging survival of transplanted cells, tissues or organs are provided. In particular, methods of prolonging survival of allotransplanted cells, tissues or organs are provided. These methods are directed to using one or more immunosuppressants in addition to an inhibitor of complement activity. Use of one or more immunosuppressants and an inhibitor of complement activity in the manufacture of one or more medicaments or medicament packages is also provided. Such medicaments or medicament packages are useful in prolonging survival of an allograft in a subject mammal.

In certain embodiments, the inhibition of complement activity is effected by chronic administration of a drug directed against complement C5. A preferred drug that inhibits complement activity is an antibody specific to one or more components of complement, for example, C5. In certain preferred embodiments, the antibody inhibits the cleavage of C5 and thereby inhibits the formation of both C5a and C5b-9. The antibody may be, e.g., a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), an antibody fragment (e.g., Fab), a single chain antibody, an Fv, or a domain antibody. The recipient is also treated with one or more immunosuppressive drugs, for example, cyclosporin A.

In certain embodiments, either an MHC mismatched recipient (i.e., a mammalian recipient of an MHC mismatched allograft), a presensitized recipient or an ABO mismatched recipient (i.e., a mammalian recipient of an AMB mismatched allograft) is treated. In this model, the recipient is again chronically treated with a complement inhibitor, preferably an anti-C5 monoclonal antibody, together with immunosuppressive drugs, preferably a chronic administration of cyclosporin A and a short-term administration of cyclophosphamide. This triple therapy results in extended graft survival in the presensitized allotransplant recipient.

The present disclosure also provides methods of prolonging survival of an allograft in a mammalian recipient by administering to the recipient agents that modulate the level and/or ratio of subclasses and/or isotypes of anti-donor immunoglobulins (Ig) in the recipient. In

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certain embodiments, an agent that reduces the level of anti-donor IgG1 in the recipient is preferred. In certain embodiments, an agent that increases the level of anti-donor IgG2a and/or IgG2b in the recipient is preferred. In certain embodiments, an agent that reduces the ratio of anti-donor IgG1/anti-donor IgG2a or IgG2b in the recipient is preferred.

The present disclosure also provides a method of prolonging survival of an allograft in a second mammalian recipient using an allograft that has been accommodated in a first mammalian recipient (i.e., the allograft has prolonged survival in the first recipient). The present disclosure further provides an allograft that is resistant to anti-donor antibodies in a mammalian recipient, and the allograft is prepared from a first recipient that has accommodated the allograft. In preferred embodiments, the first recipient has accommodated the allograft by receiving a treatment as described herein, such a triple therapy treatment involving administering to the first recipient a drug that inhibits complement activity and two immunosuppressive agents.

Further provided are pharmaceutical packages. A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation is stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized,

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chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show anti-donor antibody levels in presensitized versus unsensitized recipients under different treatments.

Figures 2A and 2B show comparison between triple therapy using anti-C5 antibody, CsA and CyP in presensitized allograft recipients and combination therapy using only anti-C5 antibody and CsA in presensitized allograft recipients. Figure 2A compares heart-allograft survival in various recipients under different treatments as indicated. Figure 2B shows histology and immunohistology, for example, for lymphocyte infiltration in heart allografts of recipients in different groups.

Figure 3 shows blocked terminal complement activity by anti-C5 antibody as compared to immunosuppressive agents.

Figures 4A-4D compare levels of anti-donor antibodies in presensitized recipients of allografts under monotherapy with anti-C5 antibody alone, double combination therapy with anti-C5 antibody and CsA, and triple combination therapy with anti-C5 antibody, CsA and CyP.

Figures 5A and 5B show change of ratios of IgG isotypes in allograft recipients that were untreated or under different treatments.

Figure 6 shows high-level expression of Bcl-2 and Bcl-xl proteins in long-term surviving heart grafts as compared to heart grafts of untreated animals.

Figure 7 shows improved second transplantation (re-transplantation) of an accommodated graft from a first transplantation recipient.

Figure 8 shows results from re-transplantation experiments.

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Figure 9 shows results from re-transplantation experiments.

DETAILED DESCRIPTION

Overview: Rejection of Transplants or Grafts

Hyperacute rejection occurs within minutes to hours after transplant and is due to preformed antibodies to the transplanted tissue antigens. It is characterized by hemorrhage and thrombotic occlusion of the graft vasculature. The binding of antibody to endothelium activates complement, and antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis and lead to vascular occlusion, the result being that the grafted organ suffers irreversible ischemic damage (Abbas et al., 2000). Hyperacute rejection is often mediated by preexisting IgM alloantibodies, e.g., those directed against the ABO blood group antigens expressed on red blood cells. This type of rejection, mediated by natural antibodies, is the main reason for rejection of xenotransplants. Hyperacute rejection due to natural IgM antibodies is no longer a major problem with allografts because allografts are usually selected to match the donor and recipient ABO type. Hyperacute rejection of an ABO matched allograft may still occur, usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well defined alloantigens expressed on vascular endothelial cells. Such antibodies may arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies (this prior exposure being referred to as "presensitization"). Abbas et al., 2000.

Acute rejection is a process of vascular and parenchymal injury mediated by T cells, macrophages, and antibodies that usually begins after the first week of transplantation. Abbas et al., 2001. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis. Both $CD4^+$ and $CD8^+$ cells may contribute to acute rejection. The destruction of allogeneic cells in a graft is highly specific and a hallmark of $CD8^+$ cytotoxic T lymphocyte killing. Abbas et al., 2000. $CD4^+$ T cells may be important in mediating acute graft rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in grafts, with some evidence available that indicates that $CD4^+$ T cells are sufficient to mediate acute rejection. Abbas et al., 2000. Antibodies can also mediate acute rejection after a graft recipient mounts a humoral immune

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response to vessel wall antigens and the antibodies that are produced bind to the vessel wall and activate complement. Abbas et al., 2000.

Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. Graft arterial occlusion may occur as a result of the proliferation of intimal smooth muscle cells (Abbas et al., 2000). This process is called accelerated or graft arteriosclerosis and can develop in any vascularized organ transplant within 6 months to a year after transplantation.

For a transplant to be successful, the several modes of rejection must be overcome. Multiple approaches are utilized in preventing rejection. This may require administration of immunosuppressants, often several types to prevent the various modes of attack, e.g., inhibition of T-cell attack, antibodies, and cytokine and complement effects. Prescreening of donors to match them with recipients is also a major factor in preventing rejection, especially in preventing hyperacute rejection. Immunoadsorption of anti-HLA antibodies prior to grafting may reduce hyperacute rejection. Prior to transplantation the recipient or host may be administered anti-T cell reagents, e.g., the monoclonal antibody OKT3, Anti-Thymocyte Globulin (ATG), cyclosporin A, or tacrolimus (FK 506). Additionally, glucocorticoids and/or azathioprine may be administered to the host prior to transplant. Drugs used to aid in preventing transplant rejection include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

Allografts are rejected in part by the activation of T cells. The transplant recipient mounts a rejection response following $CD4^+$ T cell recognition of foreign antigens in the allograft. These antigens are encoded by the major histocompatibility complex (MHC). There are both Class I and Class II MHC molecules. In humans the class I MHC molecules are HLA-A, HLA-B, and HLA-C. The class II MHC molecules in humans are called HLA-DR, HLA-DQ and HLA-DP. In mice the class I MHC molecules are H-2K, H-2D and H-2L and the class II MHC molecules are I-A and I-E. When $CD4^+$ T cells bind the foreign MHC antigens they are activated and undergo clonal proliferation. The activated T cells secrete cytokines which aid in activating monocytes/macrophages, B cells and cytotoxic $CD8^+$ T

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cells. The activated monocytes/macrophages release agents which result in tissue damage, the B cells produce alloantibodies which lead to complement mediated tissue destruction, and the $CD8^+$ T cells kill graft cells in an antigen-specific manner through induction of apoptosis and cell lysis.

Immunosuppressive Agents

The numerous drugs utilized to delay graft rejection (i.e., to prolong their survival) work in a variety of ways. Immunosuppressive agents are widely used. See Stepkowski, 2000, for a review of the mechanism of action of several immunosuppressive drugs. Cyclosporin A is one of the most widely used immunosuppressive drugs for inhibiting graft rejection. It is an inhibitor of interleukin-2 or IL-2 (it prevents mRNA transcription of interleukin-2). More directly, cyclosporin inhibits calcineurin activation that normally occurs upon T cell receptor stimulation. Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) enabling it to enter the nucleus and bind to interleukin-2 promoter. By blocking this process, cyclosporin A inhibits the activation of the $CD4^+$ T cells and the resulting cascade of events which would otherwise occur. Tacrolimus is another immunosuppressant that acts by inhibiting the production of interleukin-2.

Rapamycin (Sirolimus), SDZ RAD, and interleukin-2 receptor blockers are drugs that inhibit the action of interleukin-2 and therefore prevent the cascade of events described above.

Inhibitors of purine or pyrimidine biosynthesis are also used to inhibit graft rejection. These prevent DNA synthesis and thereby inhibit cell division including the ability of T cells to divide. The result is the inhibition of T cell activity by preventing the formation of new T cells. Inhibitors of purine synthesis include azathioprine, methotrexate, mycophenolate mofetil (MMF) and mizoribine (bredinin). Inhibitors of pyrimidine synthesis include brequinar sodium and leflunomide. Cyclophosphamide is an inhibitor of both purine and pyrimidine synthesis.

Yet another method for inhibiting T cell activation is to treat the recipient with antibodies to T cells. OKT3 is a murine monoclonal antibody against CD3 which is part of the T cell receptor. This antibody inhibits the T cell receptor and suppresses T cell activation.

Numerous other drugs and methods for delaying allotransplant rejection are known to and used by those of skill in the art. One approach has been to deplete T cells, e.g., by irradiation. This has often been used in bone marrow transplants, especially if there is a

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partial mismatch of major HLA. Administration to the recipient of an inhibitor (blocker) of the CD40 ligand-CD40 interaction and/or a blocker of the CD28-B7 interaction has been used (U.S. Patent 6,280,957). Published PCT patent application WO 01/37860 teaches the administration of an anti-CD3 monoclonal antibody and IL-5 to inhibit the Th1 immune response. Published PCT patent application WO 00/27421 teaches a method for prophylaxis or treatment of corneal transplant rejection by administering a tumor necrosis factor-α antagonist. Glotz et al. (2002) show that administration of intravenous immunoglobulins (IVIg) can induce a profound and sustained decrease in the titers of anti-HLA antibodies thereby allowing a transplant of an HLA-mismatched organ. Similar protocols have included plasma exchanges (Taube et al., 1984) or immunoadsorption techniques coupled to immunosuppressive agents (Hiesse et al., 1992) or a combination of these (Montgomery et al., 2000). Changelian et al. (2003) teach a model in which immunosuppression is caused by an oral inhibitor of Janus kinase 3 (JAK3) which is an enzyme necessary for the proper signaling of cytokine receptors which use the common gamma chain (γc) (Interleukins-2, -4, -7, -9, -15, -21), the result being an inhibition of T cell activation. Antisense nucleic acids against ICAM-1 have been used alone or in combination with a monoclonal antibody specific for leukocyte-function associated antigen 1 (LFA-1) in a study of heart allograft transplantation (Stepkowski, 2000). Similarly, an anti-ICAM-1 antibody has been used in combination with anti-LFA-1 antibody to treat heart allografts (Stepkowski, 2000). Antisense oligonucleotides have additionally been used in conjunction with cyclosporin in rat heart or kidney allograft models, resulting in a synergistic effect to prolong the survival of the grafts (Stepkowski, 2000). Chronic transplant rejection has been treated by administering an antagonist of TGF-β which is a cytokine involved in differentiation, proliferation and apoptosis (U.S. Patent Application Publication US 2003/0180301).

Complement and Transplant/Graft Rejection

The role of complement in transplant rejection is well known. This is especially true in the case of xenotransplantation, but complement also plays a role in allotransplant rejection. For review, see Platt and Saadi, 1999. One aspect of complement's role is that ischemia-reperfusion injury may occur at the time that an organ graft is reperfused with the blood of the recipient. Complement may also cause some manifestations of allograft rejection.

The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to

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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 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.

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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 (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

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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.

Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pretransplant crossmatch testing, this prototype of humoral rejection is now rarely observed (Regele et al., 2001). Data are now showing that humoral immune mechanisms might contribute to other types of allograft rejection (Regele et al., 2001). High levels of panel reactive antibodies indicating humoral presensitization were found to be associated with inferior kidney graft survival (Opelz, 1992), the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome (Jeannet et al., 1970; Halloran et al., 1992), and selective removal of recipient IgG by immunoadsorption reversed some rejection episodes indicating the contribution of humoral immune mechanisms to rejection (Persson et al., 1995; Böhmig et al., 2000). Complement activation within a graft might indicate antibody-mediated graft injury. The complement cleavage product C4d is a marker for activation of the antibody-dependent classical pathway. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome.

Recently increasing evidence indicates that complement activation significantly contributes to the sensitization of allograft recipients and the development of tissue injury in allografts (Platt et al., 1999). Antibodies are the most thoroughly investigated mediators of activating the classical complement pathway. Clinically, alloantibodies are known to activate complement (Baldwin et al., 2001). Halloran and Collins indicate that C4d deposition in peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies (Collins et al., 1999; Halloran, 2003). Further supporting evidence is seen in animals with complement inhibition (Pratt et al., 1996; Pruitt et al., 1991; Forbes et al., 1978) or deficiency (Pratt et al., 2000; Baurer et al., 1995) which exhibit significantly reduced inflammatory injury and lowered anti-donor immune responses. In ABMR, complement is suggested to be activated by the classical pathway and to play a key role in the pathogenesis (Collard et al., 1997). Although the role of complement in HAR or acute vascular rejection (AVR) following xenotransplantation has been well documented (Platt et al., 1999), precise mechanisms of complement in the pathogenesis of ABMR following allotransplantation has not yet been elucidated.

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The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition within the immune-mediated inflammatory response. As described above, C5a is a powerful anaphylatoxin and chemotactic factor. Cellular activation by C5a induces the release of multiple additional inflammatory mediators (Jose et al., 1983). The complement activation pathways (classical, alternative, or mannan-binding lectin pathway) ultimately lead to the formation of the cytolytic membrane attack complex C5b-9 (Kirschfunk, 2001), which can mediate both direct tissue injury by cell lysis, and proinflammatory cell activation at sublytic doses (Saadi et al., 1995; Papadimitriou et al., 1991). Therefore, blocking both C5a and C5b-9 generation may be required for the optimal inhibition of complement-mediated inflammatory response following transplantation. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b, preserving C3b-mediated opsonization of pathogenic microorganisms as well as solubilization and clearance of immune complexes (Liszewski, 1993).

The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction. In addition, complement inactivation by a functionally blocking anti-C5 monoclonal Ab (mAb) prevented HAR in xenotransplantation models (Kroshus et al., 1995; Wang et al., 1999).

Methods of delaying allotransplant rejection by administration of complement inhibitors have been tested. Published PCT patent application WO 92/10205 discloses the use of a combination of cyclosporin and a soluble complement receptor (sCR1) to inhibit rejection of a cardiac allotransplant in a presensitized rat model. Complement receptor 1 binds complements C3b and C4b. Soluble forms of complement receptor 1 occur naturally or can be generated via recombinant DNA procedures. These soluble complement receptors have inhibited in vitro the consequences of complement activation (U.S. Patent 6,057,131). In WO 92/10205, rats, which had been presensitized to the cardiac allograft they were receiving, were administered cyclosporin A intramuscularly at 10 mg/kg/day beginning two days prior to transplant and continued until the time of graft rejection. Additionally, soluble complement receptor 1 (sCR1) was administered as a single intravenous bolus at 15 mg/kg immediately prior to reperfusion of the graft. Control animals with no drug treatment had the

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graft rejected at an average of 3.8 days. Those administered cyclosporin A alone rejected the grafts at an average of 57 days (this was quite variable with two rats rejecting quickly at 2 and 4 days and a third rat rejecting at 166 days). Rats administered sCR1 alone rejected the grafts at an average of 44 days. Those rats administered the combination of cyclosporin A and sCR1 rejected the grafts at an average of 147 days. The combination of chronic cyclosporin A and single bolus sCR1 was seen to result in a synergistic effect greatly prolonging the time until graft rejection. Earlier studies by Pruitt and Bollinger (1991) used a similar model of a presensitized rat allograft to show that administration of sCR1 alone to inactivate complement resulted in increased time before graft rejection.

Sims et al. (U.S. Patent 5,135,916) suggest using inhibitors of complement, e.g., CD59 or antibodies against C7 or C9 to block the formation of the C5b-9 complex, to treat the vascular endothelium of organs and tissues to be transplanted. This would prevent the C5b-9 initiated cell necrosis. The C5b-9 inactivators would be added to the perfusate or storage medium to protect the vascular lining cells from ongoing complement activation during in vitro storage. Additionally the organ or tissue would be protected from the cytolytic and thrombotic effects arising from complement activation initiated upon transplantation, thereby circumventing complement mediated acute rejection. Sims et al. (U.S. Patent 5,573,940 and U.S. Patent 6,100,443) also teach a method of expressing CD59 in the transplanted tissue or organ to protect the transplanted organ from rejection. This can be accomplished by transfecting the cells being transplanted.

Although the several drugs developed to date in combination with methods of prescreening donors and recipients to match the donor allograft to the recipient have over time increased the average length of time of survival of allografts, many allografts are nonetheless rejected during the life-time of the recipient. In general, the prior art advances have mainly been directed to overcoming acute graft rejection. Further, the role of activated terminal complement components in antibody-mediated allograft rejection has not been examined using inhibitors that specifically target the complement cascade at the C5 protein level. The methods described herein and as exemplified in the Examples advance the allotransplant art by inhibiting chronic rejection of allografts, in particular, allografts in a presensitized recipient. New methods are presented for further prolonging allograft survival by using a proper combination of immunosuppressive drugs in combination with a chronic administration of a complement inhibitor.

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Methods and Uses

The methods disclosed herein are used to prolong allograft survival. The methods generally include administering an inhibitor of complement activity in combination with one or more immunosuppressants.

Suitable complement inhibitors are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C5a, C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). Proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and prevents it from being cleaved into C5a and C5b thereby preventing the formation not only of C5a but also the C5b-9 complex. Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

Suitable immunosuppressants include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

An allograft can include a transplanted organ, part of an organ, tissue or cell. These include, but are not limited to, heart, kidney, lung, pancreas, liver, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells.

At least part of the reason for the failure of allografts is that one response by the recipient of an allograft is the activation of complement. This results in the formation of C5a and C5b-9 which are potent proinflammatory molecules which aid in causing graft failure. Without wishing to be bound by any proposed theory, Applicants theorized that inhibiting the

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formation of C5a and C5b-9 or inhibiting C5a and C5b-9 which was present would aid in preventing graft failure. Furthermore, it was theorized that so long as the allograft is present, the recipient will continue to attempt to mount an immune response against the graft, and this response will include attempts to produce C5a and C5b-9. If not prevented, this complement response will lead to acute vascular rejection in the short term and could contribute to chronic graft rejection in the long term. Prior art methods of using inhibitors of complement activity were limited to administering these inhibitors only at the time of transplant. This was helpful in preventing acute rejection, but as the results disclosed herein illustrate, improved results are obtained by administration of such inhibitors for a longer term. This long-term administration aids in preventing a chronic rejection of the allograft as opposed to only aiding in preventing an acute rejection. The result is a longer term survival of the allograft as compared to either not administering an inhibitor of complement activity or administering such an inhibitor only at the time of transplant of the allograft. Although very commonly it is desirable that the allograft will survive for the remaining lifetime of the recipient, there are times when the allograft is needed only for a shorter length of time, e.g., a bridge organ to bridge the time until the recipient's own organ can recover on its own, at which time the allograft will no longer be needed. The length of time such a graft will be needed will vary, but will usually be longer than the time at which acute rejection would occur and may be long enough for chronic rejection to occur. This period of desired survival for a bridge graft may be several months, e.g., six months.

To prove that long-term inhibition of complement activity will prolong allograft survival, experiments were performed in which complement activation was inhibited in a chronic fashion and not merely at the time of transplant. Chronic treatment means treatment during an extended period up to the lifetime of the allograft. This can be daily treatment but is not limited to daily treatment. Chronic treatment will maintain an effective amount of the drug in the allograft recipient. For example, a preferred method is to include the anti-C5 monoclonal antibody eculizumab in the treatment. In studies of persons suffering from paroxysmal nocturnal hemoglobinuria (PNH), eculizumab has been administered at a dose of 900 mg/patient once every 12-14 days. This dosing has been found to completely and consistently block terminal complement activity and has greatly inhibited the symptoms of PNH (Hillmen et al., 2004). The administered dose is able to block the effects of complement for approximately two weeks before the eculizumab is inactivated or removed from the body. Therefore, a chronic treatment of eculizumab may be, e.g., the administration

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of 900 mg to the allograft recipient once every two weeks for the remaining life-time of the patient. Similarly, other drugs can be delivered chronically as needed, whether this is on a daily basis or another schedule is required to maintain an effective amount of the drug in the allograft recipient. Because it is well known that graft rejection can be caused by more than just complement activation, e.g., by T cell activity, the experiments included immunosuppressants such as cyclosporin to further aid in preventing graft rejection.

A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., 1987). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, a single-chain antibody, a domain antibody and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., 1986), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies when the recipient of the allograft is a human.

Administration and Formulations

Administration of the inhibitor of complement activity is performed according to methods known to those of skill in the art. These inhibitors are administered preferably before the time of allograft transplantation or at the time of transplantation with administration continuing in a chronic fashion. These inhibitors can additionally be administered during a rejection episode in the event such an episode does occur.

The present disclosure also provides uses of a drug that inhibits complement activity and an immunosuppressive agent in the manufacture of a medicament or medicament package. Such medicament or medicament package is useful in prolonging allograft survival in a recipient, in particular, chronic survival of the allograft. In preferred embodiments, the

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medicament or medicament package is formulated and prepared such that it is suitable for chronic administration to the recipient of the allograft, for example, stable formulations are employed. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for concurrent administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for sequential (in either order) administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft.

A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

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In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form. A formulated product of the present disclosure can be included within a container, typically, for example, a vial, cartridge, prefilled syringe or disposable pen. A doser such as the doser device described in U.S. Patent 6,302,855 may also be used, for example, with an injection system of the present disclosure.

A "stable" formulation is one in which the drug (e.g., an antibody) or agent therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. For example, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8 °C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

An isotonic reconstituted formulation is preferable in certain embodiments. By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

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A "lyoprotectant" is a molecule which, when combined with a drug (e.g., antibody) of interest, significantly prevents or reduces chemical and/or physical instability of the drug (e.g., antibody) upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the drug (e.g., antibody) in the presence of the lyoprotecting amount of the lyoprotectant, the drug (e.g., antibody) essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl parahen, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

Accordingly, a stable lyophilized antibody formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation

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can be reconstituted to generate a stable reconstituted formulation having an antibody concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the antibody concentration in the pre-lyophilized formulation. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, the reconstituted formulation can be stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8 °C. for at least about 30 days. See U.S. Patent 6,821,515. In certain embodiments, the reconstituted formulation is isotonic.

When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where a subject patient requires frequent administrations of the drug or antibody and/or agent to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

The present disclosure also provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of an antibody and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable.

An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a desirable antibody concentration in the reconstituted formulation. The article of manufacture may further comprise a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

An injection system of the present disclosure may employ a medication delivery pen as described in U.S. Patent 5,308,341. Medication delivery pens have been developed to facilitate the self-administration of medication. A medication of the present disclosure can be a drug that inhibits complement activity, for example an antibody specific to complement C5,

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and/or an immunosuppressive agent. One medication delivery pen includes a vial holder into which a vial of insulin or other medication may be received. The vial holder is an elongate generally tubular structure with proximal and distal ends. The distal end of the vial holder includes mounting means for engaging a double-ended needle cannula. The proximal end also includes mounting means for engaging a pen body which includes a driver and dose setting apparatus. A disposable medication containing vial for use with the prior art vial holder includes a distal end having a pierceable elastomeric septum that can be pierced by one end of a double-ended needle cannula. The proximal end of this vial includes a stopper slidably disposed in fluid tight engagement with the cylindrical wall of the vial. This medication delivery pen is used by inserting the vial of medication into the vial holder. A pen body then is connected to the proximal end of the vial holder. The pen body includes a dose setting apparatus for designating a dose of medication to be delivery by the pen and a driving apparatus for urging the stopper of the vial distally for a distance corresponding to the selected dose.

The user of the pen mounts a double-ended needle cannula to the distal end of the vial holder such that the proximal point of the needle cannula pierces the septum on the vial. The patient then selects a dose and operates the pen to urge the stopper distally to deliver the selected dose. The dose selecting apparatus returns to zero upon injection of the selected dose. The patient then removes and discards the needle cannula, and keeps the prior art medication delivery pen in a convenient location for the next required medication administration. The medication in the vial will become exhausted after several such administrations of medication. The patient then separates the vial holder from the pen body. The empty vial may then be removed and discarded. A new vial can be inserted into the vial holder, and the vial holder and pen body can be reassembled and used as explained above.

Accordingly, a medication delivery pen generally has a drive mechanism for accurate dosing and ease of use. A dosage mechanism such as a rotatable knob allows the user to accurately adjust the amount of medication that will be injected by the pen from a prepackaged vial of medication. To inject the dose of medication, the user inserts the needle under the skin and depresses the knob once as far as it will depress. The pen may be an entirely mechanical device or it may be combined with electronic circuitry to accurately set and/or indicate the dosage of medication that is injected into the user. See U.S. Patent 6,192,891.

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The present disclosure also presents controlled-release or extended-release formulations suitable for chronic and/or self-administration of a medication.

The various formulations can be administered to a patient in need of treatment (e.g., a recipient of an allograft) with the medication (e.g., an antibody of the present disclosure and at least one immunosuppressive agent) by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

In certain embodiments, a formulation is administered to the patient by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease® and Genject® devices); injector pens (such as the GenPen®); needleless devices (e.g. MediJector® and BioJector®); and subcutaneous patch delivery systems.

The present methods and uses are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. The following abbreviations are used herein: ABMR, antibody-mediated rejection; ACHR, accelerated humoral rejection; ACR, acute cellular rejection; AVR, acute vascular rejection; CsA, cyclosporin; CyP, cyclophosphamide; HAR, hyperacute rejection; MCP-1, monocyte chemotactic protein 1; MST, mean survival time; POD, postoperative day.

Example 1

Methods

Animals and Immunosuppressive Drugs Male adult C3H (H-2^k) mice and BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, Maine) weighing 25-30 g were chosen as donors and recipients, respectively. In the groups receiving immunosuppression, the recipients were injected with CsA (15 mg/kg/day, s.c., daily from day 0 to endpoint rejection or until day 100), or with CyP (40 mg/kg/day, i.v., on day 0 and 1), or with anti-C5 mAb (clone BB5.1, Alexion Pharmaceuticals Inc., 40 mg/kg/day, i.p., day 0–2, followed by twice a week, day 0-60). Animals were housed under conventional conditions at the Animal Care Facility,

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University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. Olfert et al., 1993.

Skin Presensitization Full-thickness skin grafts taken from C3H donors were cut into square pieces of 1×1 cm^2 and transplanted onto the back of the BALB/c recipients' thorax one week prior to heart transplantation from the same donors. Rejection was defined as complete necrosis of the skin grafts.

Abdominal and Cervical Cardiac Transplantation Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomen of presensitized BALB/c recipients by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava. In the groups with re-transplantation, second heart grafts harvested from either naive C3H mice or long-term surviving presensitized BALB/c recipients were transplanted into the cervical area of the recipients carrying a long-term surviving first abdominal heart graft by anastomosing the donor aorta and recipient carotid artery, and the donor pulmonary artery and recipient external jugular vein (end-to-side). The heart grafts were monitored daily until rejection unless otherwise indicated and rejection was defined as complete cessation of pulsation.

Experimental Groups Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: Group 1, mice with no treatment; Group 2, mice treated with CsA; Group 3, mice treated with CyP; Group 4, mice treated with CsA plus CyP; Group 5, mice treated with anti-C5 mAb; Group 6, mice treated with anti-C5 mAb plus CsA; Group 7, mice treated with anti-C5 mAb plus CyP; Group 8, mice treated with anti-C5 mAb in combination of CsA and CyP. When cardiac impulses were no longer palpable or at POD100, the grafts were removed for routine histology, immunohistochemistry and western blot analysis, serum samples were collected for flow cytometric analysis and complement hemolytic assay. Five additional animals were placed and sacrificed in groups 6 and 8 on POD3 (MST for groups 1-5, 7) to allow for comparisons at a uniform time point. Serum samples were also collected on POD 11, 21, 28 and 60 in Group 8 for detecting the sequential changes of anti-donor antibody levels and complement activity. In addition, when triple therapy treated presensitized recipients carried a first heart graft for 100 days, they were re-transplanted with a second heart. A naive C3H heart or a 100-day surviving C3H heart from another presensitized BALB/c recipient was used as the second heart. Eight animals were included in each re-transplant group.

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Graft Histology Tissue samples were fixed in 10% buffered formaldehyde. Specimens were then embedded in paraffin, and sectioned for H&E staining. The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist. Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage and lymphocyte infiltration. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change.

Immunohistochemistry Four micrometer sections were cut from tissue samples embedded in Tissue-Tek O.C.T gel (Optimum Cutting Temperature, Skura Finetek, Torrance, CA) mounted on gelatin-coated glass microscope slides and stained by a standard indirect avidin-biotin immunoperoxidase staining method using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Specimens were stained for $CD4^+$ and $CD8^+$ cells with biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.1.2, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and biotin-conjugated rat anti-mouse CD8 mAb (clone 53-6.7, Pharmingen, Franklin Lakes, NJ), respectively. Intragraft monocyte/macrophage infiltration was detected by staining with biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Mouse IgG and IgM deposition in grafts was detected using biotin-conjugated goat anti-mouse-IgG and goat anti-mouse-IgM (Cedarlane). For identification of complement deposition, sections were serially incubated with goat anti-C3 or anti-C5 polyclonal Abs (Quidel, San Diego, CA), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated-streptavidin (Zymed Laboratories, South San Francisco, CA). Slides were washed with phosphate-buffered saline between steps, and examined under light microscopy. Negative controls were performed by omitting the primary antibodies. The immunostaining was scored in five high-power fields of each section, and five independent experiments were performed. The sections of immunoperoxidase staining were graded from 0 to 4+ according to the staining intensity: 0, negative; 1+, equivocal; 2+, weak staining; 3+, moderate staining; and 4+, very intensive staining.

Flow Cytometry The circulating anti-donor specific IgG and IgM antibodies were evaluated in the recipient serum by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Glotz et al., (1993); Tyan et al. (1994). Briefly, C3H mouse splenocytes were isolated and incubated at 37 °C for 30 minutes with serum from naive control and experimental groups. To stain for total IgG, IgG1, IgG2a, IgG2b and IgM, the cells were washed and incubated with FITC-conjugated goat antibody specific for the Fc portion of mouse IgG or with

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phycoerythrin-conjugated goat antibody specific for the μ-chain of mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or with FITC-conjugated goat anti-mouse IgG1 (CALTAG Laboratories, Burlingame, CA), or with FITC-conjugated goat anti-mouse IgG2a (CALTAG), or with FITC-conjugated goat anti-mouse IgG2b (CALTAG). After 1 hour of staining at 4 °C, the cells were washed with PBS, resuspended at 5×10^6/mL, and analyzed by flow cytometry for mean channel fluorescence intensity, which represents the antibody-binding reactivity.

Complement Hemolytic Assay The purified anti-C5 mAb was serially diluted twofold (175-0.1 μg/ml) in GVB^{2+} buffer (gelatin Veronal-buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1.8 mM sodium barbital) and added in triplicate (50 μl/well) to a 96-well plate. BALB/c mouse serum was diluted to 40% v/v with GVB^{2+} buffer and added (50 μl/ml) to the rows of the same 96-well plate such that the final concentration of BALB/c mouse serum in each well was 20%. The plate was then incubated at room temperature for approximately 30 min while chicken erythrocytes were prepared. Chicken erythrocytes were washed 5×1 ml with GVB^{2+} buffer and resuspended to a final concentration of 5×10^7/ml in GVB^{2+}. Four milliliters of the chicken erythrocytes were sensitized by adding anti-chicken RBC polyclonal antibody (Intercell Technologies, Hopewell, NJ, 0.1% v/v) and the cells were incubated at 4 °C for 15 min with frequent vortexing. The cells were then washed 2×1 ml with GVB^{2+} and resuspended to a final volume of 2.4 ml in GVB^{2+}. The chicken erythrocytes (30 μl/well, 2.5×10^6 cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000×g for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at OD 415 nm using a microplate reader and the percentage of hemolysis was determined using this formula:

$$\%\ \text{hemolysis} = 100 \times \frac{(\text{OD sample}) - (\text{OD } GVB^{2+} \text{ control})}{(\text{OD 100\% lysed control}) - (\text{OD } GVB^{2+} \text{ control})}$$

with 100% lysed control obtained by the addition of 100 μl GVB^{2+} containing 0.1% NP-40 to the 30 μg/ml of chicken erythrocytes as prepared above.

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Western Blot Analysis Sonication of frozen heart samples was performed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) at 4 °C for 1 minute at 10-second intervals, followed by microcentrifugation at 13,000 rpm for 10 minutes at 4 °C. Clarified supernatants were immediately quantitated in triplicate for protein content using Detergent-compatible protein assay kit (BIO-RAD). Heart lysates (10μg protein/well) were separated on NuPAGE, 4-12% gradient Bis-Tris gels and MES buffer system (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size; Invitrogen) using a semi-dry transfer apparatus (BIO-RAD). Membranes were cut appropriately at the correct molecular weights to allow the development of the blots with two different primary antibodies per blot such that each blot was exposed to a test antibody and an internal control antibody to insure equal sample loading. The test primary antibodies including anti-Bcl-2 (N-19) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) and anti-Bcl-XS/L (M-125) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) were used to detect intragraft expression of Bcl-2 and Bcl-xl proteins. Anti-calsequestrin rabbit polyclonal sera (Calbiochem) were used as internal control primary antibody (Kobayashi et al., 1999). Detection of primary antibody binding was performed as previously described (Arp et al., 1996) by exposing washed incubated blots to a polyclonal goat anti-rabbit IgG fraction conjugated to horseradish peroxidase (HRP; Roche Laboratories) and then appropriately developed by exposure to enhance chemiluminescence for HRP-conjugated antibodies (Roche Laboratories).

Statistical Analysis The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the rank-log test. Histological and immunohistological findings were analyzed using the Mann-Whitney U test. Flow cytometric data and western blot data were analyzed using one-way ANOVA. Differences with p values less than 0.05 were considered significant.

Example 2

Presensitization with C3H donor skin graft induces antibody-mediated ACHR in heart allografts of BALB/c recipients.

To develop a suitable small animal model that mimics presensitized patients in the clinic and to study ABMR, a novel, fully MHC-mismatched mouse ABMR model has been developed through presensitization of mouse recipients. In this model, BALB/c recipients were presensitized with C3H donor skin grafts one week prior to heart transplantation from the same donor. Seven days after donor skin presensitization, serum level of anti-donor IgG,

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but not IgM antibody was markedly elevated and reached to a peak level in the presensitized BALB/c recipients (Figure 1A). Heart transplantation from same donor was then performed in these highly sensitized recipients. Without immunosuppression, C3H heart grafts were rapidly rejected in 3.1 ± 0.4 days by ACHR, characterized by severe thrombosis, hemorrhage and infarction (Figure 1B-a). In contrast, same heart grafts in unsensitized BALB/c recipients (with mean survival time, MST of 8.2 ± 0.8 days) show the normal histology on post-operative day (POD) 3 (Figure 1B-b). When compared to unsensitized BALB/c recipients at the same day, heart grafts in presensitized animals revealed massive IgG antibody and complement (C3 and C5) deposition, but minimal $CD4^+$ and $CD8^+$ cell infiltration (Table 1). Furthermore, circulating anti-donor IgG levels in presensitized recipients were significantly higher than those of unsensitized same recipients receiving a heart graft on POD3 ($P< 0.01$, Figure 1C). However, anti-donor IgM remained at very low levels both in circulation (Figure 1C) and in heart grafts (Table 1) and it showed no significant difference between unsensitized and presensitized recipients. In addition, normal levels of complement hemolytic activity were shown in both presensitized and unsensitized heart recipients without treatment (Figure 1D). These data indicate that this is an ideal transplant model to study ABMR in presensitized recipients in which complement plays an important role in the pathogenesis.

Table 1.

Comparison of immunohistological changes of C3H heart allografts in unsensitized and presensitized BALB/c recipients on POD3

Groups	Unsensitized	Presensitized
IgG	1+	4+
IgM	1+	1+
C3	2+	3+
C5	2+	3+
CD4	0	1+
CD8	0	1+

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Grades for immunoperoxidase staining: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb in combination with CsA and CyP prevents ABMR and achieves indefinite heart allograft survival in presensitized mouse recipients.

Complement has been shown to play an important role in ABMR. However, the inhibitory effect of functionally blocking terminal complement cascade at the C5 level in highly sensitized recipients is unknown. In the study presented herein, the presensitized model was used to study the efficacy of anti-C5 mAb either alone or combined with CsA and /or CyP in prevention of ABMR. As presented in Figure 2A, treatment with either CsA or CyP or the two drugs in combination did not prevent ABMR and grafts were rejected in 3.0 ± 0.0 days, 3.3 ± 0.5 days and 3.5 ± 0.6 days, respectively with typical pathological features of ACHR including intravascular thrombosis and interstitial hemorrhage (Figure 2B-b, c, d), which were indistinguishable from heart grafts in untreated presensitized BALB/c recipients (Figure 2B-a). Anti-C5 monotherapy or combined with CyP was not able to improve graft survival and heart grafts were rejected by ACHR (Figure 2B-e, f) in 3.5 ± 0.6 days and 3.2 ± 0.4 days, respectively (Figure 2A). Although the combination therapy of anti-C5 mAb and CsA, the protocol capable of inducing long-term heart allograft survival in unsensitized animals, marginally prolonged graft survival in this presensitized model, heart grafts were also rejected by severe humoral rejection with vasculitis, thrombosis, hemorrhage and minimal cell infiltration (Figure 2B-g) in 11.9 ± 1.8 days (Figure 2A). In contrast, triple therapy of anti-C5 mAb in combination of CsA and CyP achieved indefinite heart graft survival over 100 days (Figure 2A) in presensitized animals ($P<0.01$ vs. the animals without treatment or treated with either monotherapy or two drugs in combination) with no evidence of rejection (Figure 2B-h). In this presensitized mouse model, as shown in Table 2, only minor intragraft $CD4^+$ and $CD8^+$ cell infiltration was observed in the recipients that rejected their heart grafts within 3 days. However, the number of these T cells was slightly increased if heart grafts survived longer in anti-C5 mAb plus CsA-treated recipients at the time of rejection (POD11) and in triple therapy-treated recipients at early stages of graft survival (e.g. POD11). Furthermore, with continuous treatment of CsA in the triple therapy group, $CD4^+$ and $CD8^+$ cell infiltration was inhibited in long-term surviving heart grafts on POD60 and 100. In addition, moderate intragraft Mac-1^+ cell infiltration, including monocytes and macrophages, was found in untreated and CsA-, CyP- or CsA plus CyP-treated animals,

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while the infiltration of these cells was significantly reduced in anti-C5 mAb treated animals (Table 2). These results indicate that functionally blocking anti-C5 mAb enables the use and efficacy of conventional immunosuppressive agents, thereby preventing ABMR and achieving indefinite heart graft survival in presensitized recipients.

Table 2.

Grades for immunoperoxidase staining of heart allografts

in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Untreated	3	3+	3+	1+	1+	3+	4+	1+
CsA	3	3+	3+	1+	1+	3+	4+	1+
CyP	3	3+	3+	1+	1+	3+	3+	1+
CsA+CyP	3	3+	3+	1+	1+	3+	3+	1+
Anti-C5mAb	3	3+	0	1+	1+	2+	4+	1+
Anti-C5mAb+CsA	11	3+	0	2+	2+	2+	4+	1+
Anti-C5mAb+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	11	3+	0	2+	2+	1+	3+	1+
Anti-C5mAb+CsA+CyP	60	3+	0	1+	1+	0	2+	1+
Anti-C5mAb+CsA+CyP	100	3+	2+	0	0	0	2+	1+

Grades: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb completely inhibits total complement hemolytic activity and local C5 deposition in presensitized recipients receiving a heart allograft.

Anti-C5 mAb was previously shown to block the cleavage of complement protein C5 into the proinflammatory molecules C5a and C5b-9 (Kroshus et al., 1995), and to completely and consistently block terminal complement activity in mice (Wang et al., 1999). In the current study, terminal complement activity was measured by assessing the ability of

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recipient mouse sera to lyse antibody presensitized chicken erythrocytes and was compared at the same time-point (POD3). Treatment of mice with either CsA or CyP or the two drugs in combination had no effect on terminal complement activity, while treatment with anti-C5 mAb either alone or combined with CsA or/and CyP completely inhibited this activity (Figure 3; $P<0.01$, vs. naive and untreated animals, as well as CsA-, CyP-, or CsA plus CyP-treated animals). In addition, sera obtained from anti-C5 mAb treated animals at several earlier time points showed similarly diminished hemolytic activity, suggesting that serum terminal complement was inhibited throughout the treatment period. Furthermore, local C5 deposition in heart grafts was completely prevented in the anti-C5 mAb treated presensitized recipients, but not in untreated, or CsA-, CyP- and CsA plus CyP-treated presensitized animals (Table 2). As predicted, treatment with anti-C5 mAb did not prevent C3 deposition in the grafts (Table 2). These results suggest anti-C5 therapy completely blocks total complement activity after cardiac allografting in highly sensitized recipients.

Long-term surviving heart grafts in presensitized animals are resistant to humoral injury in the presence of low level of anti-donor antibodies and complement- a situation of accommodation.

To further investigate the role of anti-C5 mAb in humoral rejection, anti-donor alloantibody levels were measured in recipient sera by flow cytometry and intragraft antibody deposition by using immunostaining techniques in different groups. Figure 4A shows that on POD3 untreated presensitized BALB/c recipients had high levels of circulating anti-donor IgG antibodies. When presensitized recipients receiving either monotherapy or two drugs in combination, CsA and/or CyP partially down-regulated circulating anti-donor IgG levels, while treatment with anti-C5 mAb either alone or combined with CsA or CyP did not further affect anti-donor antibody levels at the same day. In contrast, with triple therapy of anti-C5 mAb, CsA and CyP, a high level of circulating anti-donor IgG was gradually downregulated and reached a low level on POD60, thereafter remaining at this level until day 100 (Figure 4B). Similar to levels of circulating antibodies in the different treatment groups, Table 2 shows that strong deposition of anti-mouse IgG was present in the rapidly rejected heart grafts of presensitized animals with no treatment or treated with monotherapy or two drugs in combination therapy. Interestingly, with triple therapy, IgG antibody deposition was gradually attenuated to a mild level in the long-term surviving heart grafts on POD 100 (Figure 4C-a, Table 2). In this model, IgM remained at very low levels in either circulation (Figure 4A, B) or transplanted heart grafts (Figure 4C-b, Table 2) in presensitized recipients

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with or without treatment. In addition, treatment with anti-C5 mAb eliminated complement activity to an undetectable level until day 60, followed by a progressive recovery to predepletion levels on POD 100 after discontinuation of anti-C5 therapy in presensitized mouse recipients receiving triple therapy (Figure 4D). Furthermore, intragraft C5 deposition was also detected in 100-day surviving presensitized animals (Table 2). These data demonstrate that ongoing transplant accommodation occurs in triple therapy treated presensitized recipients despite the presence of anti-graft antibodies and complement activation.

Anti-C5 mAb in combination with CsA and CyP reduces the IgG1/IgG2a ratio and leads to a shift in IgG subclass to IgG2b in recipients with accommodated grafts.

To determine whether anti-C5 mAb-based triple therapy would induce a shift in IgG subclass, which may be associated with accommodation, serum levels of anti-donor IgG subclasses of IgG1, IgG2a and IgG2b were compared between untreated recipients and the recipients with accommodated heart graft. Sera from untreated recipients contained predominant IgG1 isotype, indicated by a high ratio of IgG1/IgG2a (Figure 5A). In contrast, a significant reduction in the ratio of IgG1/IgG2a was observed in the recipients carrying accommodated grafts (Figure 5A, $P<0.01$). Furthermore, presensitized recipients with the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared to the same recipients with rejected grafts (Figure 5B, $P<0.01$). In addition, the pattern of IgG isotypes in the recipients treated with either monotherapy or two drugs in combination is indistinguishable from that of untreated animals. These data indicate that anti-donor IgG1 isotype may be associated with graft rejection, while production of anti-donor IgG2b subclass may function as a protective antibody and plays an important role in the induction of accommodation.

Anti-C5 mAb in combination with CsA and CyP induces intragraft Bcl-2 and Bcl-xl expression in highly sensitized mouse recipients.

To determine whether a causal relationship exists between intragraft expression of protective proteins and graft resistance to humoral injury in this model, western blot analysis was employed to detect proteins of interest in heart graft tissues from highly sensitized mouse recipients. Long-term surviving heart grafts were found to express high levels of Bcl-2 and Bcl-xl proteins on POD100, and these proteins were detected as early as 12 days after heart transplantation in highly sensitized recipients receiving anti-C5 mAb-based triple therapy

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(Figure 6). In contrast, there were no Bcl-2 and Bcl-xl proteins expressed on heart grafts of untreated animals (Figure 6) or animals treated with either monotherapy or two drugs in combination therapy. This result suggests that graft resistance to humoral injury in indefinite surviving animals is associated with the protection provided by Bcl-2 and Bcl-xl proteins in this presensitized model.

Presensitized recipients with an accommodating first heart graft accept a second accommodated heart graft but reject a second naive heart graft from the same donors.

The ability of accommodated grafts to resist rejection has not been tested directly under pathophysiological conditions where naive grafts undergo rejection following allotransplantation. In this model, to determine whether presensitized recipients with an accommodating first heart graft will accept a second accommodated graft but reject a second naive graft, we performed re-transplantation scenarios. After the accommodated C3H heart grafts have survived to the 100-day point, the time at which low levels of alloantibodies were detected (Figure 4B) and complement activity has returned to pretreatment levels (Figure 4D), in presensitized BALB/c recipient treated with anti-C5 mAb-based triple therapy, these recipients received a second heart graft. Specifically, either a naive (Figure 7A) or a 100-day accommodated C3H heart (Figure 7B) from another presensitized BALB/c recipient was transplanted into the neck of the presensitized recipients carrying an accommodating first C3H heart. These recipients rejected a second naive heart at 6.6 ± 1.1 days (Figure 8A) with severe AVR (Figure 8B-a) while the first heart continued to survive. In contrast, when the accommodated hearts that had been already surviving for 100 days in different presensitized mice were used as second grafts, these grafts were accepted by the presensitized recipients carrying an accommodating first heart graft (Figure 8A). There was no sign of rejection in those accommodated second heart grafts 90 days after second transplantation (Figure 8B-b). These data indicate that accommodated grafts become resistant to the effects of anti-donor antibodies and complement that normally mediate allograft rejection in these presensitized recipients. Furthermore, the fact that the host of the accommodated graft rejected a new graft suggests that accommodation involves changes to the graft.

Presensitized recipients being treated with CsA reject accommodated heart grafts.

Another re-transplantation was performed to determine whether accommodation in this presensitized model would be caused by the changes in the grafts and/or the recipients. Specifically, after C3H heart grafts have been accommodated in presensitized BALB/c mice

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for 100 days, the accommodated heart graft will then be re-transplanted into a second presensitized BALB/c recipient being treated with CsA alone (Figure 7C), a therapy that can prevent cellular rejection but cannot prevent accelerated humoral rejection of a fresh C3H heart in presensitized recipients. The accommodated C3H heart grafts were rapidly rejected in CsA treated presensitized BALB/c recipients. After re-transplantation, the pathology in accommodated heart grafts was changed from normal (Figure 9A) to severe ACHR with massive interstitial hemorrhage but few cell infiltrates (Figure 9B). In addition, high levels of anti-donor IgG and normal levels of complement hemolytic activity in these recipients receiving an accommodated C3H heart were similar to those of CsA treated presensitized recipients receiving a naive C3H heart. This result further indicates that accommodation induced by anti-C5 mAb-based triple therapy can originate from mechanisms involving changes not only to the graft, but also to the recipient.

Example 3

Acute Vascular Rejection in a Heart Transplantation Model

Experiments were performed to determine whether inclusion of an inhibitor of formation of terminal complement would attenuate acute vascular rejection and whether the use of such an inhibitor in conjunction with an immunosuppressant would achieve long-term allograft survival. In this set of experiments an anti-C5 monoclonal antibody was used in conjunction with cyclosporin. The model used was an allograft heterotopic heart transplant from C3H mice into BALB/c mice. This model is a stringent acute vascular rejection model with the C3H and BALB/c mice being strongly MHC mismatched. The transplantations and other methods were performed as described in Wang et al. (2003).

Heterotopic Cardiac Transplantation

Intra-abdominal heterotopic cardiac transplantation was performed as previously described by Wang et al. (2003). Briefly, a median sternotomy was performed on the donor, and the heart graft was slowly perfused in situ with 1.0 ml of cold heparinized Ringer's lactate solution through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were transected, and the graft was removed from the donor. The graft was then revascularized with end-to-side anastomoses between the donor's pulmonary artery and the recipient's inferior vena cava as well as the donor's aorta and the recipient's abdominal aorta using 11-0 nylon suture. The beating of the grafted heart was monitored daily by direct abdominal palpation.

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The degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology. In certain instances, mice in which the graft was still functioning were sacrificed to perform histology.

Results

Mice (male 8-12 week old mice weighing 25 – 30 g) were split into six experimental groups with six to eight mice per group. Transplant occurred on day 0. Histological changes were checked at the endpoint (the endpoint being graft failure) or in some cases a mouse was sacrificed prior to graft failure. The dosage of BB5.1 which was administered (40 mg/kg body weight three times per week) was known from prior studies to completely inhibit terminal complement activity.

Group 1' (control) - mice were administered 0.75 mL of saline intraperitoneally on days -1, 0, 1 and 2. Subsequently these mice were treated with 0.75 mL of saline intraperitoneally three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 2' (cyclosporin A alone) - mice were administered 15 mg/kg body weight of cyclosporin A subcutaneously on a daily basis beginning at day 0 (day of transplant) until the endpoint.

Group 3' (anti-complement antibody alone) - mice were administered the anti-mouse C5 antibody BB5.1 (Frei et al., 1987) at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 4' (anti-complement antibody until day 14 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intravenously on days -1 through day 14 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint. Note that this differs from the other groups in that the BB5.1 was administered intravenously and on a daily basis.

Group 5' (anti-complement antibody until day 28 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until day 28 and were also administered

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cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint.

Group 6' (anti-complement antibody chronically until 100 days plus cyclosporin) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until 100 days and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until 100 days.

The results of this experiment are shown in Tables 3 and 4. Table 3 shows the survival time for the grafts. Table 4 sets forth the histological scores.

Table 3

Allograft Survival

Group (Treatment)	Individual Survival (days)	Mean Survival Time (days)
1'. Saline	8, 8, 8, 8, 8, 9	8.3 ± 0.5
2'. Cyclosporin A	14, 15, 15, 16, 16, 16, 17	15.5 ± 1.1
3'. BB5.1	7, 8, 8, 8, 8, 9	8.0 ± 0.6
4'. BB5.1 until day 14 + cyclosporin A	35, 38, 43, 45, 46, 47	42.3 ± 4.8
5'. BB5.1 until day 28 + cyclosporin A	77, 80, 80, 81, 82	80 ±1.9
6'. BB5.1 until day 100 + cyclosporin A	>100 days (7 mice; one sacrificed for histology)	> 100 days

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Table 4

Median Scores of Histological Changes of Heart Allografts at Necropsy

Groups	Vasc*	Infarc	Lymph	Throm	Hemo	Fibrin	PMN
1'. Saline (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
2'. Cyclosporin A (endpoint)	2.0	1.0	2.0	3.0	2.0	2.0	3.0
3'. BB5.1 (endpoint)	2.0	1.0	2.0	2.0	1.0	0.0	0.0
4'. BB5.1 until day 14 + cyclosporin A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5'. BB5.1 28 days + Cyclosporin A (post-operative day 8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5'. BB5.1 28 days + Cyclosporin A (endpoint)	0.0	0.0	1.0	1.0	2.0	1.0	0.0
6'. BB5.1 until day 100 + Cyclosporin A (post-operative day 100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available.

*Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate

The results indicate the synergistic effects of using a complement inhibiting drug in addition to an immunosuppressant. In untreated mice the grafts were rejected in about 8 days. Use of the immunosuppressant cyclosporin A alone on a daily, chronic basis resulted

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in an increase in graft survival until approximately 15 days post-transplant. The use of the anti-C5 antibody BB5.1 to inhibit formation of terminal complement had no effect on its own, graft rejection occurring at 8 days post-transplant as in the control group (Group 1'). The combination of BB5.1 through day 28 post-transplant plus cyclosporin A showed a synergistic effect with graft survival being extended until approximately day 80. A more surprising result is that of Group 5' in which BB5.1 and cyclosporin A were each administered chronically post-transplant. In this case the graft survival was for more than 100 days (as much data as presently available). Additionally, the histological results shown in Table 4 indicate that the administration of both BB5.1 and cyclosporin A protected the graft from changes much better than either BB5.1 or cyclosporin A alone, and that the chronic administration of BB5.1 and cyclosporin A protected the graft to such an extent that even at 100 days post-transplant there were no histological changes seen in the engrafted hearts. A survival time of 100 days in these models is considered to be the gold standard. A survival of 100 days in the model is believed to indicate that there will be an indefinite survival of the allograft. When BB5.1 administration was stopped after 28 days, the grafts were protected but they did begin to show some minimal to mild histological changes by about day 80 which was the time at which graft failure occurred.

The Group 4' mice were treated differently in that they were administered BB5.1 on a daily basis by an intravenous administration. These animals became ill, showing weight loss and urine retention and were sacrificed at a time at which the grafted hearts were still beating although their function had declined. This was the first group of mice studied and it is unknown why these ill effects were seen. These ill effects were not seen when the BB5.1 was administered intraperitoneally with a schedule of three times per week. As seen below in Example 4, daily administration of BB5.1 via an intraperitoneal route did not cause ill effects. Also, intravenous administration was not necessarily the cause of the illness in these animals. Intravenous administration of eculizumab (a human equivalent antibody to BB5.1 in that it binds to human C5) has been successfully administered intravenously without ill effects to humans in a study of PNH (Hillmen et al., 2004). Complement inhibitors may be administered by other routes in addition to intravenous and intraperitoneal, with all such routes being well known by those skilled in the art.

Example 4

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Accelerated Rejection in a Presensitized Heart Transplantation Model

A second set of experiments similar to those of Example 3 was performed but the recipient mouse was presensitized to the donor organ. In these experiments, the presensitization was brought about by prior transplantation of a skin graft. In general, presensitization can occur not only as a result of having received an earlier allograft, but can also be caused by having received multiple blood transfusions or in women who have been pregnant. Besides such presensitization methods, allografts with an ABO mismatch will be rapidly attacked and rejected because of preformed antibodies to the ABO antigens unless steps are taken to prevent such an attack.

Some mice in these studies were administered cyclophosphamide in addition to BB5.1 and/or cyclosporin A. For these experiments BALB/c recipient mice were presensitized with C3H skin grafts one week prior to heart transplantation from the same donor (using the method of Pruitt and Bollinger, 1991). This model is designed to mimic presensitized transplantation in humans, especially in relation to accelerated humoral rejection. Recipient mice were split into eight groups of six to eight mice each. The treatments were as follow.

Group 1" (control) - mice (male 8-12 week old mice weighing 25 – 30 g) were administered 0.75 mL saline intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint (graft rejection).

Group 2" (cyclosporin A alone) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight beginning on day 0 (day of transplant) until the endpoint.

Group 3" (BB5.1 alone) - mice were administered the anti-mouse complement monoclonal antibody BB5.1 at a dose of 40 mg/kg body weight delivered intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint.

Group 4" (cyclophosphamide alone) - mice were administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 5" (BB5.1 plus cyclosporin A) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint.

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Group 6" (BB5.1 plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 7" (cyclosporin A plus cyclophosphamide) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 8" (BB5.1 plus cyclosporin A plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until 100 days. These mice were also administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until 100 days. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1. Two mice in this group were sacrificed at day 60 for histological studies (no rejection had yet occurred) and the four remaining mice still had not rejected their grafts by day 100.

Additionally a control group of mice which was not presensitized and received only the saline treatment as for Group 1" was tested.

The results of these experiments are shown in Tables 5 and 6. Table 5 lists survival times for the grafts and Table 6 summarizes the histological results.

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Table 5

Allograft Survival

Groups (Treatment)	Individual survival (days)	Mean survival time (days)
No presensitization	8, 8, 8, 8, 8, 9	8.3 ± 0.5*
1". One skin presensitization	3, 3, 3, 3, 3, 3, 3, 4	3.1 ± 0.4
2". Cyclosporin A	3, 3, 3, 3	3.0 ± 0.0
3". BB5.1	3, 3, 4, 4	3.5 ± 0.6
4". Cyclophosphamide	3, 3, 3, 4	3.3 ± 0.5
5". BB5.1 + Cyclosporin A	10, 10, 11, 11, 12, 12, 14, 15	11.9 ± 1.8**
6". BB5.1 + Cyclophosphamide	3, 3, 3, 3, 3, 4	3.2 ± 0.4
7". Cyclosporin A + Cyclophosphamide	3, 3, 3, 4, 4, 4	3.5 ± 0.6
8". BB5.1 + Cyclosporin A + Cyclophosphamide	> 100 days (4 mice)	> 100***

*P < 0.01 group 1" vs. no presensitization

**P < 0.01 group 5" vs. groups 1"-4" and 6"-7".

***P<0.01 group 8" vs. groups 1"-7".

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Table 6

Median Scores of Histological Changes of Heart Allografts at Necropsy

Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
No presensitization (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
1". One skin presensitization (endpoint)	0.0	4.0	1.0	4.0	3.0	0.0	0.0
2". Cyclosporin A (endpoint)	0.0	4.0	0.0	3.0	3.0	N/A	N/A
3". BB5.1 (endpoint)	2.0	2.0	2.0	2.0	3.0	N/A	N/A
4". Cyclophosphamide (endpoint)	2.0	4.0	0.0	3.0	2.0	N/A	N/A
5". BB5.1 + Cyclosporin A (post-operative day 3)	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5". BB5.1 + Cyclosporin A (endpoint)	2.0	2.0	2.0	2.0	2.0	0.0	0.0
6". BB5.1 + Cyclophosphamide (endpoint)	1.0	3.0	0.0	3.0	2.0	N/A	N/A
7". Cyclosporin A + Cyclophosphamide (endpoint)	0.0	1.0	1.0	2.0	3.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 3)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8. BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 12)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-	0.0	0.0	1.0	0.0	0.0	N/A	N/A

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operative day 60)							
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 100)	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available.

*Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate

The results shown in Table 5 indicate a difference between the presensitized mouse model and the nonpresensitized mouse model as used in Example 3. The results indicate that in the absence of presensitization, grafts are rejected in approximately 8 days in the absence of treatment with any drugs. Presensitizing the animals causes a more rapid rejection, the rejection of the graft in the presensitized animals being in approximately 3 days in the absence of any drug treatment. Treatment with either BB5.1, cyclosporin A or cyclophosphamide had no effect upon graft survival, with the grafts being rejected in approximately 3-4 days in each of these groups of animals. The combination of BB5.1 and cyclosporin A showed some effect with rejection occurring about day 12. The combination of BB5.1 and cyclophosphamide had no protective effect with rejection occurring about day 3. Similarly the combination of cyclosporin A and cyclophosphamide had essentially no protective effect with rejection occurring at 3-4 days. Very surprisingly, the combination of all three drugs (chronic administration of BB5.1 and cyclosporin plus administration of cyclophosphamide at the time of transplant) showed a highly synergistic effect with all of the mice surviving for more than 100 days. Again, a survival of 100 days in this model is considered to be the gold standard and assumes an indefinite survival.

These results as well as the histological results as shown in Table 6 indicate that the combination of chronic treatment with a complement inhibitor and an immunosuppressant such as cyclosporin A in treating a presensitized mouse results in some attenuation of accelerated rejection. Treatment of these animals additionally with cyclophosphamide at the time of transplant and on the first day after transplant results in a much greater time of survival, no rejection having been seen by at least day 100.

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It will be appreciated that the methods and compositions of the instant disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the disclosure. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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LIST OF REFERENCES

The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are incorporated herein by reference in their entirety, and for convenience, are referenced by author and date in the text and respectively grouped in the following List of References.

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The contents of all references cited herein are incorporated by reference in their entirety.

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CLAIMS

1. A method to prolong survival of an MHC mismatched allograft in a recipient mammal, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

2. The method of claim 1 wherein said mammal is a human.

3. The method of claim 2 wherein said MHC mismatched allograft is an HLA mismatched allograft.

4. The method of claim 1 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

5. The method of claim 4 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

6. The method of claim 5 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

7. The method of claim 5 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

8. The method of claim 5 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

9. The method of claim 5 wherein said antibody fragment is pexelizumab.

10. The method of claim 5 wherein said whole antibody is eculizumab.

11. The method claim 10 wherein said eculizumab is administered once every 2 weeks.

12. The method of claim 1 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

13. The method of claim 1 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

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14. The method of claim 1 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

15. The method of claim 1 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

16. The method of claim 1 wherein more than one immunosuppressive drug is administered.

17. The method of claim 1 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

18. The method of claim 17 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

19. The method of claim 1 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

20. The method of claim 1 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

21. The method of claim 1 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

22. The method of claim 1 wherein said allograft survives for the remaining life-time of said mammal.

23. The method of claim 2 wherein said allograft survives for at least six months.

24. The method of claim 2 wherein said allograft survives for at least one year.

25. The method of claim 2 wherein said allograft survives for at least five years.

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26. The method of claim 2 wherein said allograft survives for the remaining life-time of said human.

27. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

28. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

29. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

30. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

31. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

32. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

33. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

34. The method of claim 1 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

35. The method of claim 34 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

36. A method to prolong survival of an allograft in a recipient mammal presensitized to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

37. The method of claim 36 wherein said mammal is a human.

38. The method of claim 36 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

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39. The method of claim 38 wherein said drug that inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

40. The method of claim 39 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

41. The method of claim 39 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

42. The method of claim 39 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

43. The method of claim 39 wherein said antibody fragment is pexelizumab.

44. The method of claim 39 wherein said whole antibody is eculizumab.

45. The method claim 44 wherein said eculizumab is administered once every 2 weeks.

46. The method of claim 36 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

47. The method of claim 36 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

48. The method of claim 36 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

49. The method of claim 36 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

50. The method of claim 36 wherein more than one immunosuppressive drug is administered.

51. The method of claim 36 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

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52. The method of claim 51 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

53. The method of claim 51 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

54. The method of claim 36 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

55. The method of claim 36 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

56. The method of claim 36 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

57. The method of claim 36 wherein said allograft survives for the remaining life-time of said mammal.

58. The method of claim 37 wherein said allograft survives for at least six months.

59. The method of claim 37 wherein said allograft survives for at least one year.

60. The method of claim 37 wherein said allograft survives for at least five years.

61. The method of claim 37 wherein said allograft survives for the remaining life-time of said human.

62. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

63. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

64. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

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65. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

66. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

67. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

68. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

69. The method of claim 36 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

70. The method of claim 69 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

71. A method to prolong survival of an allograft in a recipient mammal which is an ABO mismatch to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

72. The method of claim 71 wherein said mammal is a human.

73. The method of claim 71 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

74. The method of claim 73 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

75. The method of claim 74 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

76. The method of claim 74 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

77. The method of claim 74 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

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78. The method of claim 74 wherein said antibody fragment is pexelizumab.

79. The method of claim 74 wherein said whole antibody is eculizumab.

80. The method claim 79 wherein said eculizumab is administered once every 2 weeks.

81. The method of claim 71 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

82. The method of claim 71 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

83. The method of claim 71 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

84. The method of claim 71 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

85. The method of claim 71 wherein more than one immunosuppressive drug is administered.

86. The method of claim 71 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

87. The method of claim 86 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

88. The method of claim 86 wherein said drug that inhibits complement activity is an antibody that inhibits cleavage of complement C5.

89. The method of claim 71 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

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90. The method of claim 71 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

91. The method of claim 71 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

92. The method of claim 71 wherein said allograft survives for the remaining life-time of said mammal.

93. The method of claim 72 wherein said allograft survives for at least six months.

94. The method of claim 72 wherein said allograft survives for at least one year.

95. The method of claim 72 wherein said allograft survives for at least five years.

96. The method of claim 72 wherein said allograft survives for the remaining life-time of said human.

97. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

98. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

99. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

100. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

101. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

102. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

103. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

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104. The method of claim 71 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

105. The method of claim 104 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

106. Use of a drug that inhibits complement activity and an immunosuppressive drug in the manufacture of a medicament or medicament package for prolong survival of an allograft in a mammal.

107. The use of claim 106, wherein more than one immunosuppressive drug is included in said medicament or medicament package.

108. The use of claim 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation suitable for concurrent administration to said mammal.

109. The use of claim 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation or formulations suitable for sequential administration to said mammal.

110. The use of claim 106, wherein said drug that inhibits complement activity is in a formulation suitable for chronic administration to said mammal.

111. The use of claim 106, wherein said immunosuppressive drug is in a formulation suitable for chronic administration to said mammal.

112. A method to prolong survival of allograft in a recipient mammal, comprising preparing an allograft from a first allograft accommodated by a first recipient mammal, wherein said first recipient mammal, after receiving said first allograft, has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

113. An allograft that has prolonged survival in a recipient mammal, wherein said allograft is prepared from a first recipient mammal and was transplanted to the first recipient mammal that has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

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114. A pharmaceutical package comprising a drug that inhibits complement activity and at least one immunosuppressive agent, wherein said drug and said agent are formulated for chronic administration.

115. The pharmaceutical package of claim 114, wherein said drug is an antibody in a lyophilized formulation comprising the antibody and a lyoprotectant.

116. The pharmaceutical package of claim 114, wherein said agent is in a lyophilized formulation comprising the agent and a lyoprotectant.

117. The pharmaceutical package of claim 114, wherein said drug and said agent are in the same lyophilized formulation comprising said drug, said agent, and a lyoprotectant.

118. The pharmaceutical package of claim 114, wherein said drug is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug in a formulation suitable for injection.

119. The pharmaceutical package of claim 114, wherein said agent is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said agent in a formulation suitable for injection.

120. The pharmaceutical package of claim 114, wherein said drug and said agent are in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug and said agent in a formulation suitable for injection.

121. The pharmaceutical package of claim 114, wherein said drug is present in unit dosage form.

122. The pharmaceutical package of claim 114, wherein said agent is present in unit dosage form.

123. A lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant, wherein said antibody formulation is suitable for chronic administration.

124. The lyophilized antibody formulation of claim 123, wherein the antibody inhibits the formation of terminal complement or C5a.

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125. The lyophilized antibody formulation of claim 123, wherein the antibody is a whole antibody or an antibody fragment.

126. The lyophilized antibody formulation of claim 125, wherein the antibody wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

127. The lyophilized antibody formulation of claim 125, wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

128. The lyophilized antibody formulation of claim 125, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

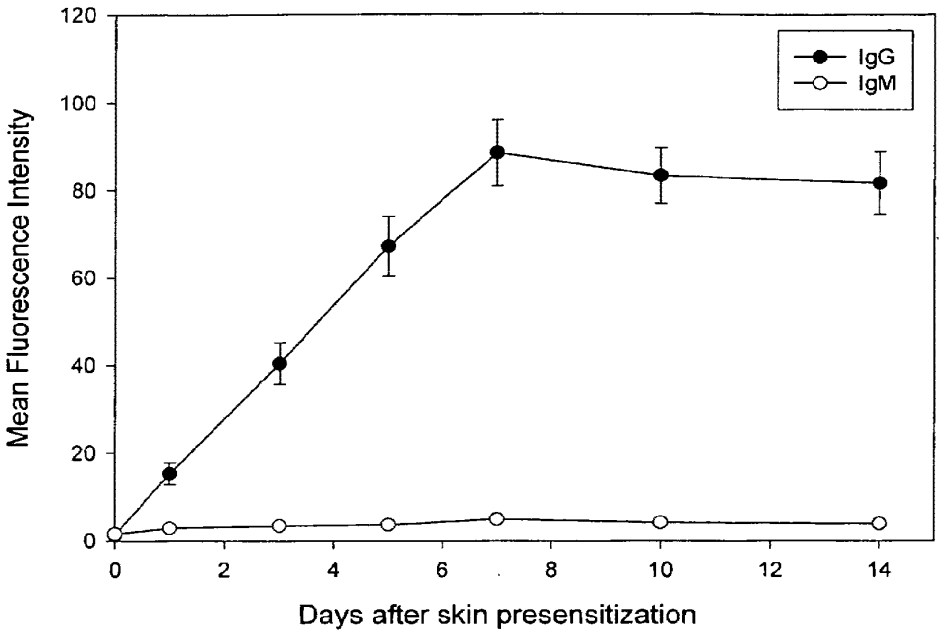
129. The lyophilized antibody formulation of claim 125, wherein said antibody fragment is pexelizumab.

130. The lyophilized antibody formulation of claim 125, wherein said whole antibody is eculizumab.

131. An injection system comprising a syringe, wherein said syringe comprises a cartridge containing an antibody that inhibits complement activity and is formulated suitable for injection.

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A



B

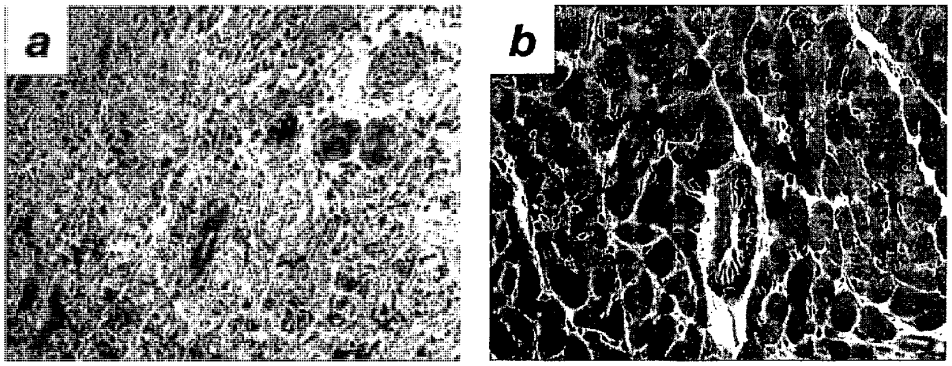


Figure 1A, B

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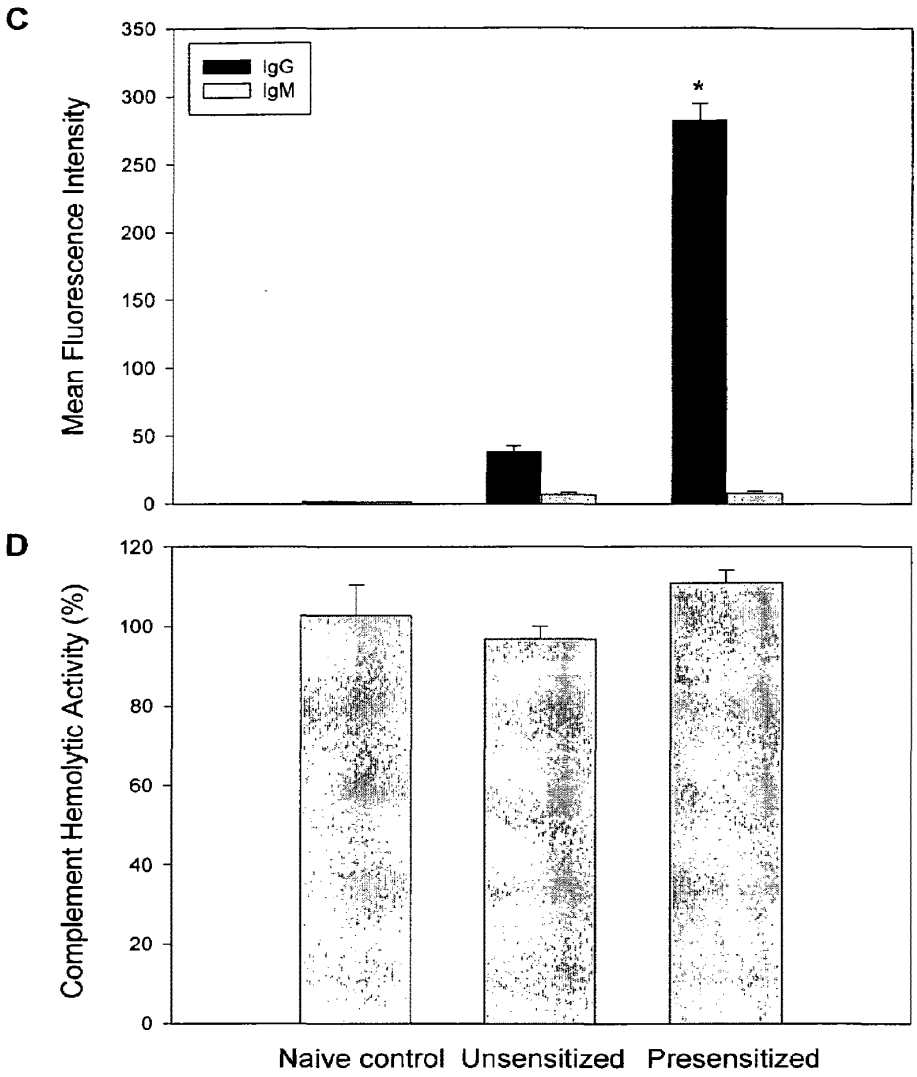


Figure 1C, D

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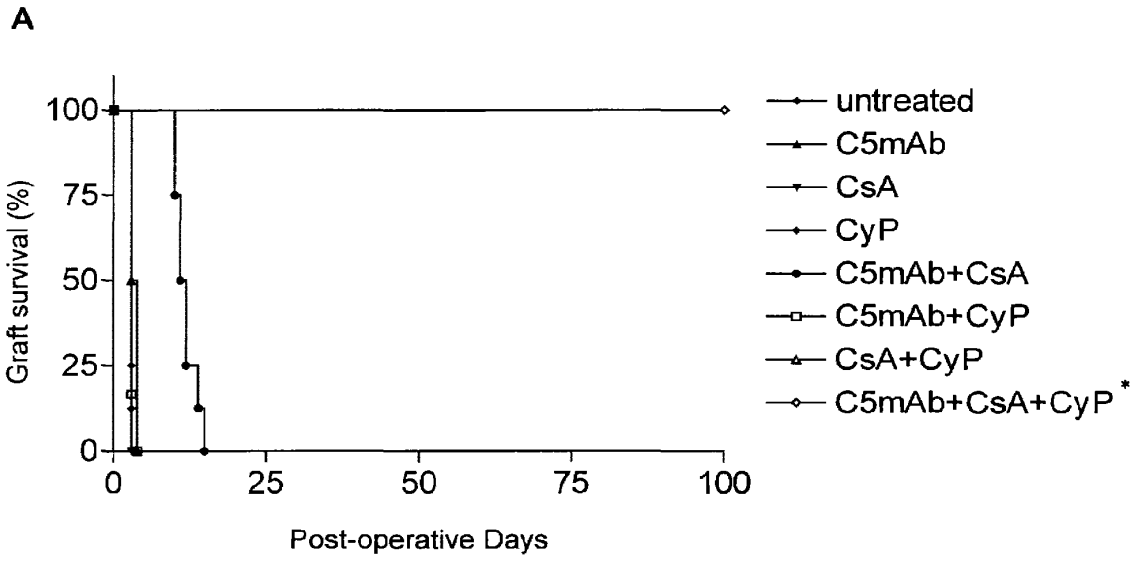


Figure 2A

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B

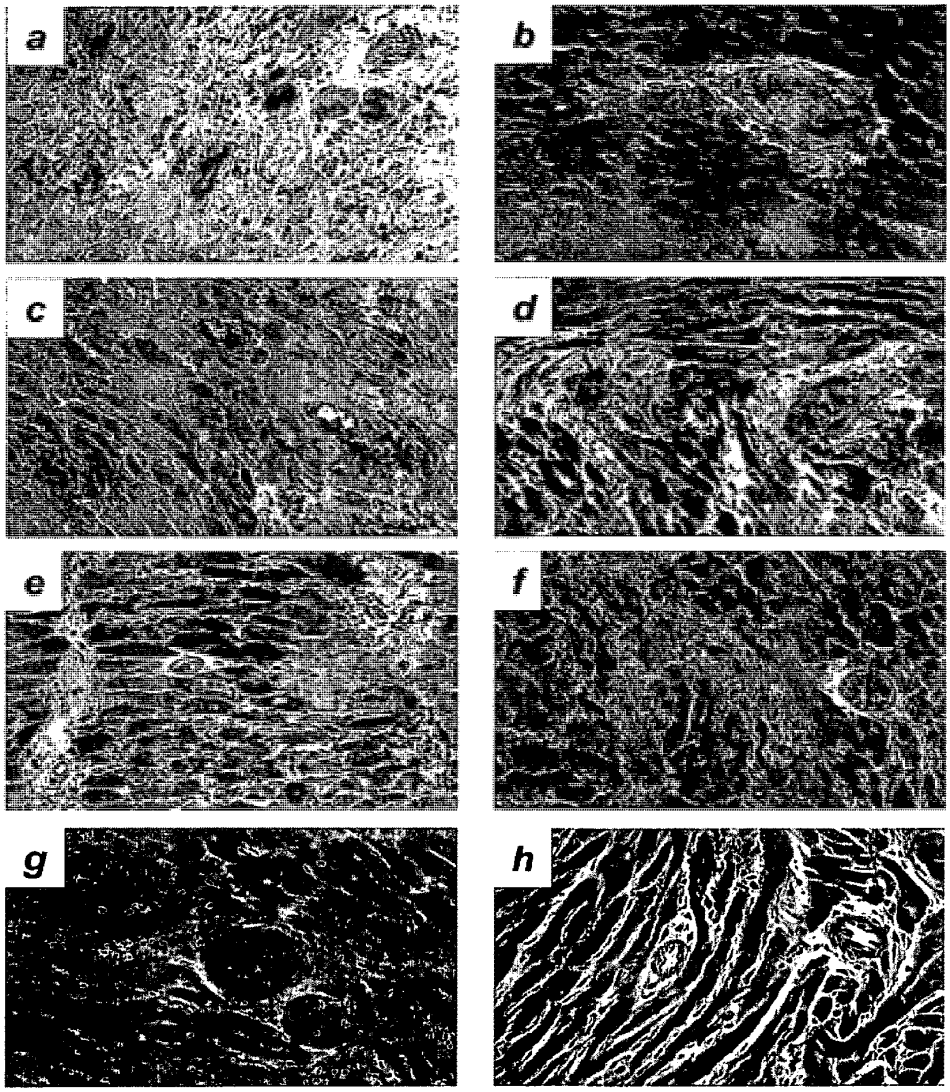


Figure 2B

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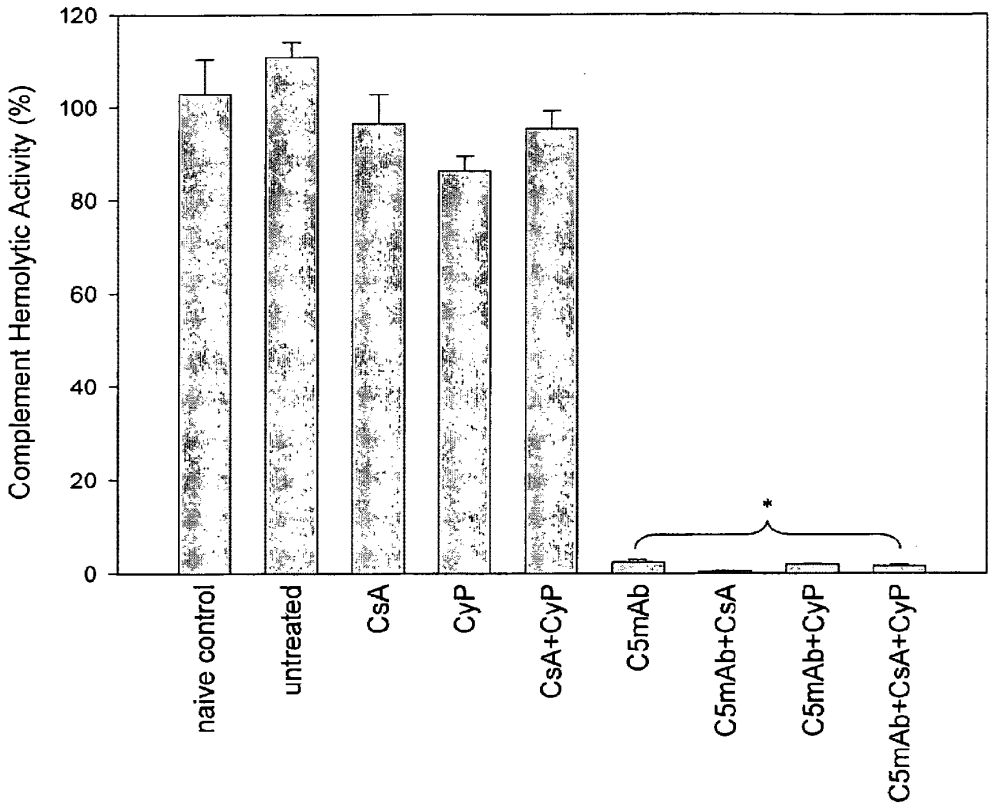


Figure 3

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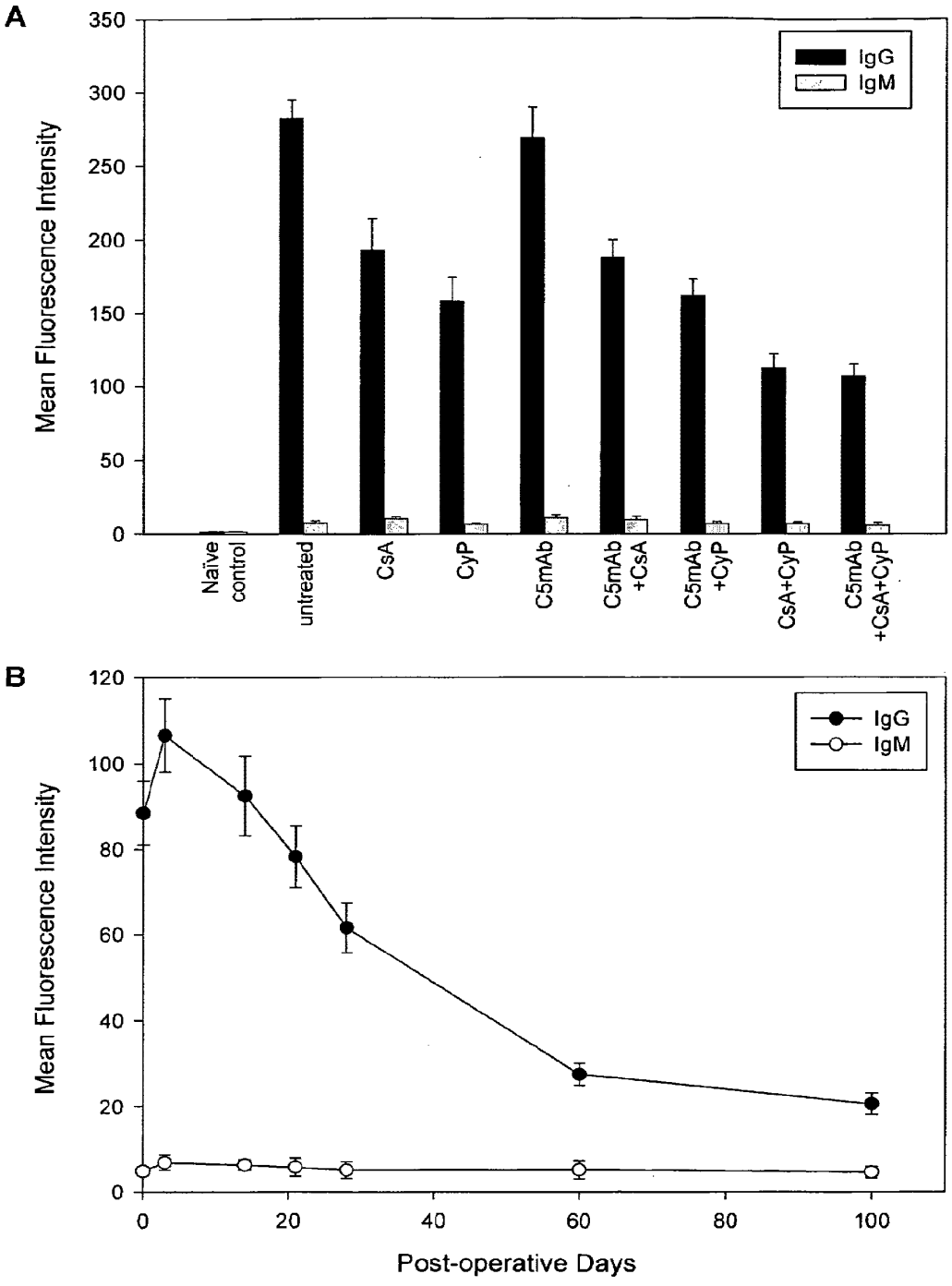
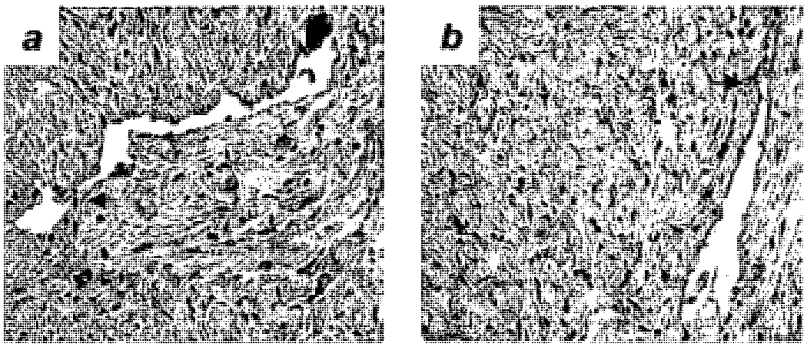


Figure 4A, B

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C



D

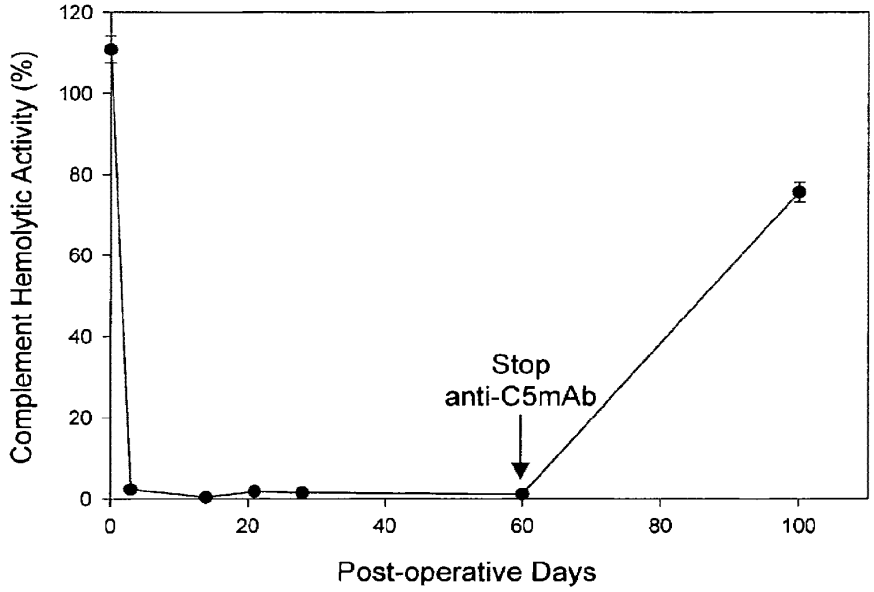


Figure 4C, D

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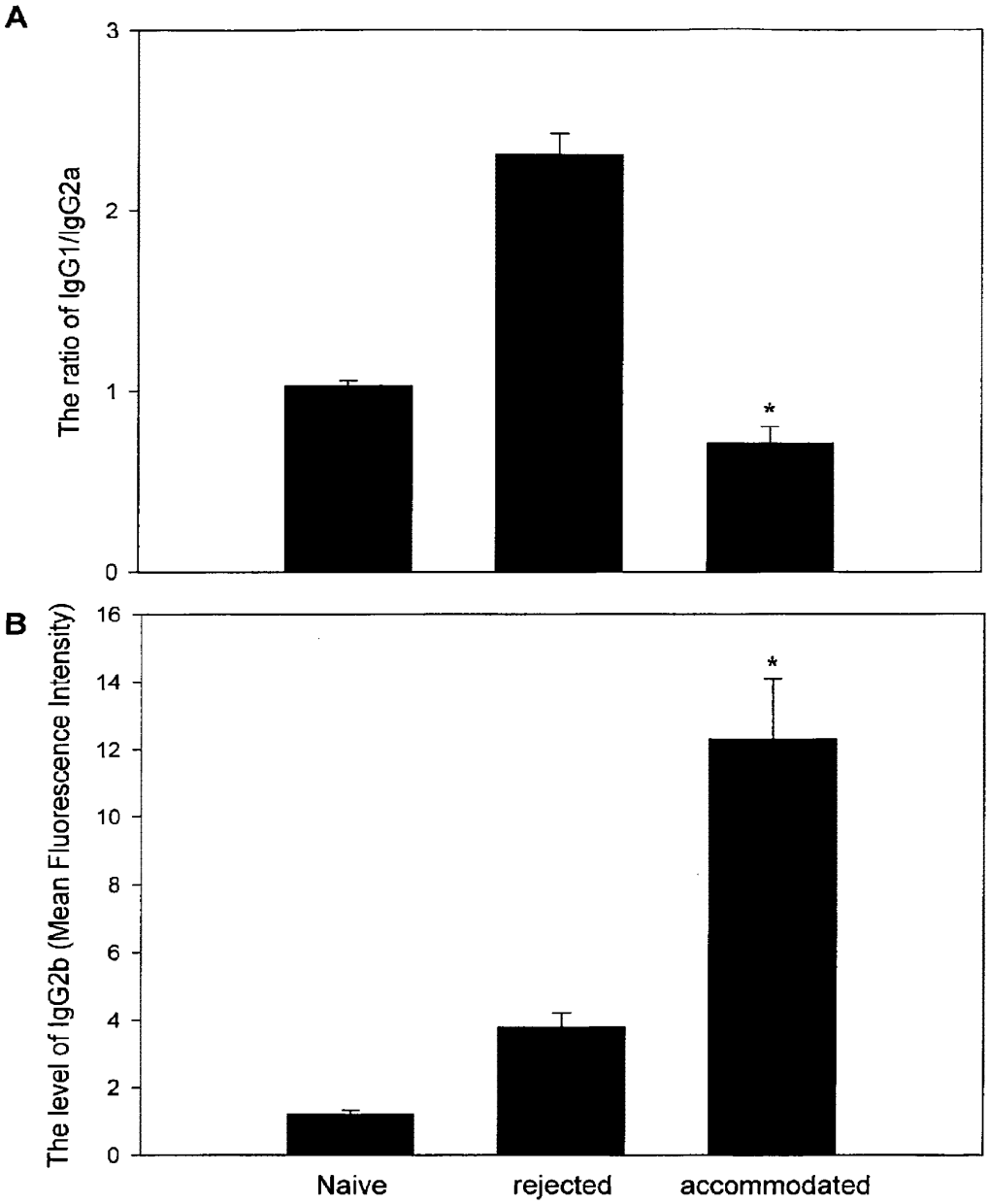


Figure 5A, B

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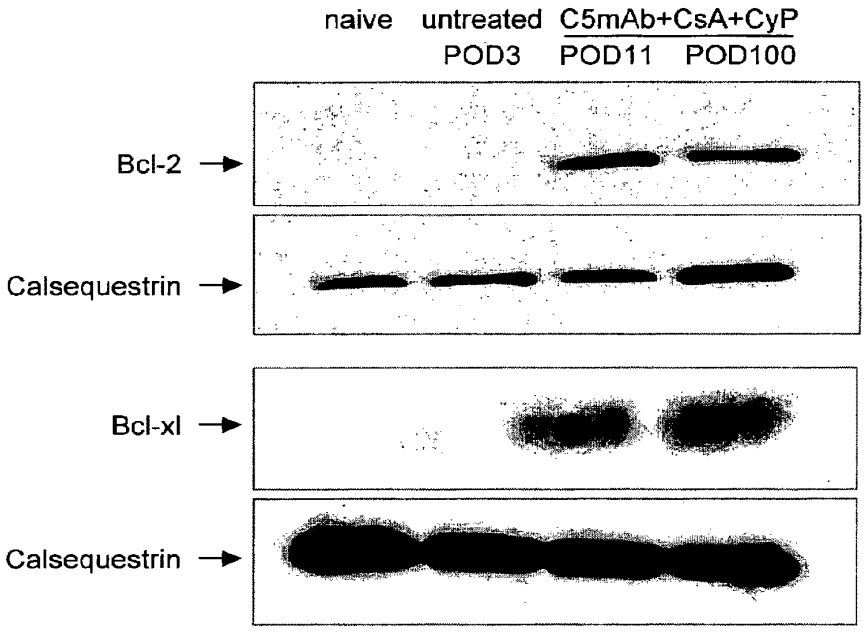


Figure 6

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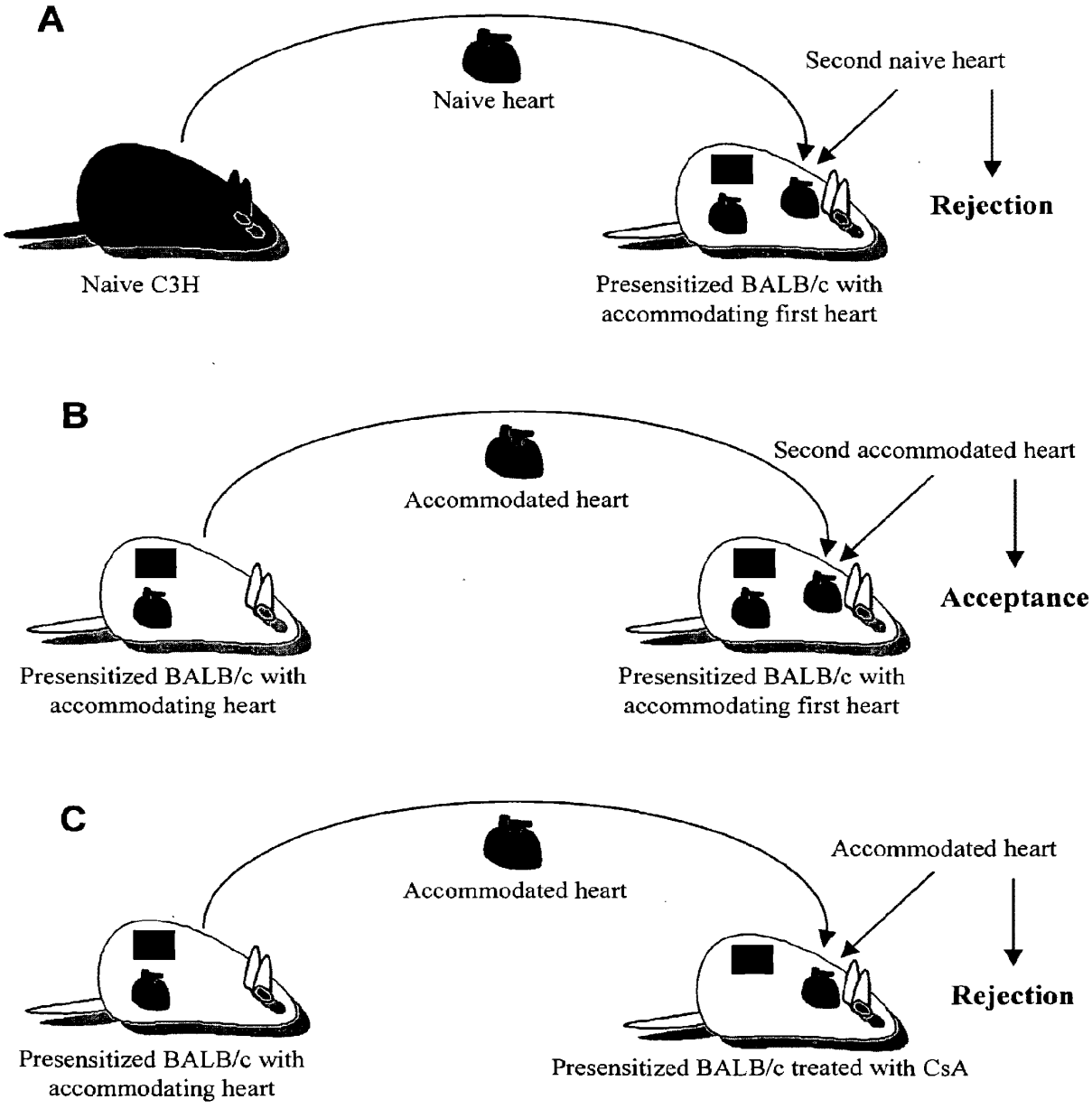
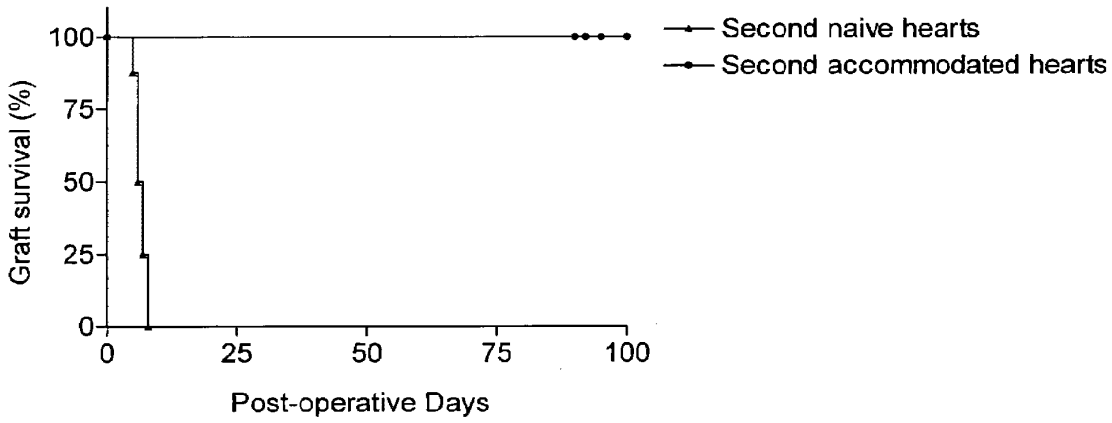


Figure 7

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A



B

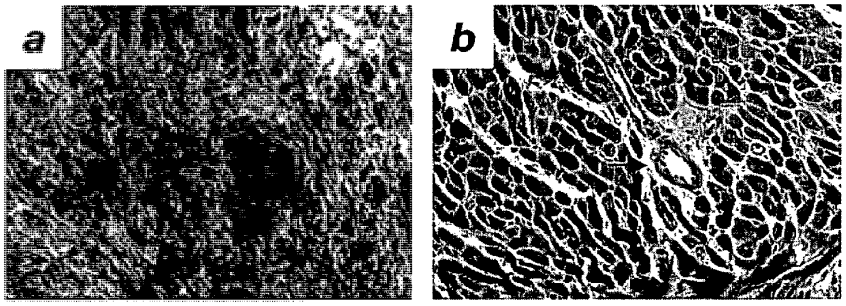


Figure 8

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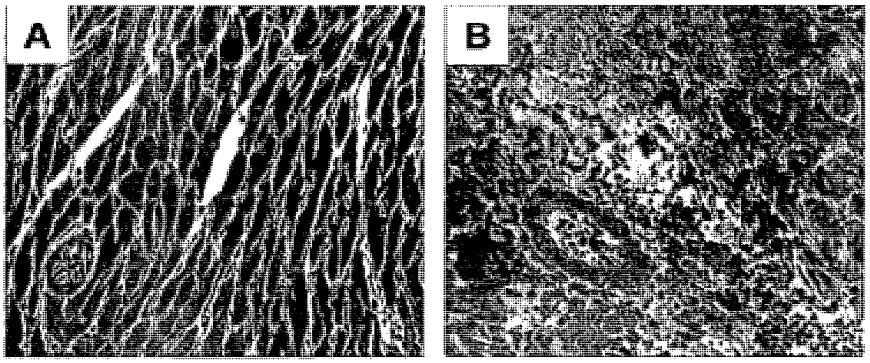


Figure 9

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **ROTHER, Russell** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US).

(74) Agents: **VARMA, Anita** et al.; Fish & Neave IP Group, Ropes & Gray LLP, One International Place, Boston, MA 02110-2624 (US).

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(54) Title: USE OF A COMPLEMENT INHIBITOR TO TREAT PATIENTS WITH SICKLE CELL DISEASE

(57) Abstract: A method of treating a sickle cell patient with a complement inhibitor is disclosed. Treatment with an inhibitor of complement activation decreases the extent of red blood cell lysis and decreases the consequential clinical sequelae.

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USE OF A COMPLEMENT INHIBITOR TO TREAT PATIENTS WITH SICKLE CELL DISEASE

FIELD OF THE INVENTION

[0001] The present invention relates to methods for the treatment of sickle cell disease. In specific embodiments, the invention relates to the use of antibodies capable of inhibiting complement as therapeutic agents to treat sickle cell disease.

BACKGROUND OF THE INVENTION

[0002] Sickle cell disease (also called sickle cell anemia) is an inherited form of anemia. The red blood cells are affected by a mutation in the beta globin gene which affects hemoglobin structure. The mutant hemoglobin causes red blood cells, which normally are smooth and round, to become hard, sticky, and shaped like sickles or crescents. These red cells tend to get stuck and block the flow of blood in small blood vessels and to lyse which in turn leads to pain, damage and a low blood count or anemia.

[0003] Sickle cell disease affects about 72,000 Americans, predominantly those of African ancestry. One in every 600 African-American births is a baby with sickle cell disease. One in every 1,000-1,400 Hispanic-American births is a baby with sickle cell disease. Those with the disease usually show some signs and symptoms after four months of age. These include: anemia because the sickle cells are fragile and break apart easily and die leaving a shortage of red blood cells to carry oxygen; periodic episodes of pain referred to as crises which develop when sickle-shaped red blood cells block blood flow through tiny blood vessels to the chest, abdomen and joints, with the pain sometimes occurring in the bones; hand-foot syndrome wherein swollen hands and feet occur when sickle-shaped red blood cells block blood flow out of the hands and feet; jaundice resulting from the liver being overwhelmed by the rapid breakdown of red blood cells; frequent infections because of damage to the spleen caused by the sickle cells; stunted growth because of a shortage of oxygen and nutrients due to a lack of healthy red blood cells; and vision problems which result from damage to blood vessels in the eye caused by sickle cells plugging those blood vessels. Other complications include stroke (caused by sickle cells blocking

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blood flow to the brain), acute chest syndrome (chest pain, fever and difficulty breathing caused by trapped sickle cells in the lungs); organ damage to not only the spleen and eyes but also the kidneys and liver due to chronic deprivation of oxygen-rich blood; and ulcers on the legs due to a lack of blood vessels to nourish the skin. Gallstones are a possible complication resulting from high levels of bilirubin due to the high level of breakdown of red blood cells. Priapism may also develop if sickle cells prevent blood flow out of an erect penis and this can lead to impotence. Persons with sickle cell disease may also experience fatigue, joint pain, breathlessness, rapid heart rate, abdominal pain, bloody urine (hematuria), excessive urination, and excessive thirst.

[0004] Presently the only cure for sickle cell disease is a bone marrow transplantation. Unfortunately very few sickle cell patients have a suitable donor for transplant. Consequently, treatment of sickle cell patients is usually aimed at avoiding crises, relieving symptoms and preventing complications. Antibiotics such as penicillin are given to children beginning when they are 2-4 months old to help prevent infections such as pneumonia. Vaccines are also given to prevent bacterial infections. Pain relievers, sometimes including narcotics, are commonly administered during a sickle crisis. Red blood cell transfusions are used to increase the number of normal red blood cells in circulation, helping to relieve anemia and its resulting symptoms and helping to prevent stroke. Blood transfusions do, however, include some risk such as a buildup of excess iron which can damage the heart, liver and other organs. Administration of supplemental oxygen via a breathing mask can be useful during an acute chest syndrome or a sickle cell crisis. Hydroxyurea, a prescription drug normally used to treat cancer, is sometimes administered to adults with severe disease. It reduces the frequency of painful crises and may reduce the need for blood transfusions. It appears to work by stimulating production of fetal hemoglobin. Patients who took hydroxyurea over a 9-year period experienced a 40% reduction in deaths. There is concern that long-term use of this drug may cause tumors or leukemia. Other drugs used to activate the production of fetal hemoglobin include 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine) which hypomethylate DNA but, as with hydroxyurea, these can be toxic carcinogens.

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[0005] Experimental treatments are being performed in an attempt to either cure or better treat sickle cell disease. Because sickle cell disease is caused by a mutation in a single gene, gene therapy is being considered as a treatment to cure the disease. However, gene therapy studies in general for other diseases have not worked well and are fraught with the possibility that the therapy will in turn cause some type of cancer due to indiscriminate insertion of the gene into the genome. Drugs being tested include butyric acid (which may increase the amount of fetal hemoglobin in the blood), clotrimazole (normally used to treat fungal infections but which may prevent loss of water from red blood cells and thereby reduce the number of sickle cells that form), and nitric oxide (a gas which helps keep blood vessels open and reduces the stickiness of red blood cells). A search of the ClinicalTrials.gov website using the search term "sickle cell" gave a listing of 55 clinical trials (including completed studies, studies no longer recruiting, studies still recruiting patients, terminated studies, and studies not yet recruiting) related to sickle cell disease or sickle cell trait. This listing is shown as Table 1.

Table 1

1. Completed	A study of the efficacy and safety of ICA-17043 (with or without hydroxyurea) in patients with sickle cell anemia. Conditions: Sickle Cell Disease; Sickle Cell Anemia
2. Completed	Mobilization and Handling of Stem Cells for Transplant from Healthy Volunteers with Sickle Cell Trait Condition: Sickle Cell Trait
3. Recruiting	Nitric Oxide Inhalation to Treat Sickle Cell Pain Crises Condition: Anemia, Sickle Cell
4. Recruiting	Evaluation of Hydroxyurea Plus L-arginine or Sildenafil to Treat Sickle Cell Anemia Condition: Sickle Cell Anemia
5. Recruiting	Natural History of Sickle Cell Disease and Other Hemolytic Disorders Conditions: Sickle Cell Anemia; Hemolytic Anemia; Hemoglobin SC Disease; Hematologic Diseases
6. Recruiting	Study of Oral ICL670 (Deferasirox) Relative to Subcutaneous Deferoxamine in Sickle Cell Disease Patients Conditions: Sickle Cell Disease; Iron Overload; Hemolytic Anemia
7. Recruiting	Collection and Storage of Umbilical Cord Stem Cells for Treatment of Sickle Cell Disease

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Conditions: Healthy; Sickle Cell Anemia

8. **Completed** Effects of Nitric Oxide and Nitroglycerin in Patients with Sickle Cell Anemia
Conditions: Chest Pain; Sickle Cell Anemia

9. **Recruiting** Secondary Pulmonary Hypertension in Adults with Sickle Cell Anemia
Conditions: Pulmonary Hypertension; Sickle Cell Anemia

10. **Completed** Nitric Oxide to Improve Blood Flow in Sickle Cell Disease
Condition: Sickle Cell Anemia

11. **Recruiting** Bone Marrow Transplantation in Treating Children With Sickle Cell Disease
Condition: Sickle Cell Anemia

12. **No longer recruiting** Hydroxyurea for the Treatment of Patients with Sickle Cell Anemia
Condition: Sickle Cell Anemia

13. **No longer recruiting** Safety of ICL670 vs. Deferoxamine in Sickle Cell Disease Patients with Iron Overload due to Blood Transfusions
Condition: Anemia, Sickle Cell

14. **No longer recruiting** Decompression Coring Versus Conservative Therapy in Patients With Avascular Necrosis of the Hip Related to Sickle Cell Disease
Conditions: Bone Avascular Necrosis; Sickle Cell Anemia

15. **Recruiting** Inhaled Nitric Oxide and Transfusion Therapy for Patients with Sickle Cell Anemia and Secondary Pulmonary Hypertension
Conditions: Sickle Cell Anemia; Pulmonary Hypertension

16. **Recruiting** Arginine Treatment of Acute Chest Syndrome (Pneumonia) in Sickle Cell Disease Patients
Conditions: Anemia, Sickle Cell; Pneumonia

17. **Recruiting** Therapeutic Application of Intravascular Nitrite for Sickle Cell Disease
Condition: Sickle Cell Anemia

18. **Recruiting** Atorvastatin Therapy to Improve Endothelial Function in Sickle Cell Disease
Condition: Sickle Cell Disease

19. **Completed** Cerebrovascular Involvement in Sickle Cell Disease - Comprehensive Sickle Cell Center
Conditions: Anemia, Sickle Cell; Blood Disease; Cerebrovascular Accident

20. **Completed** Prevention of Cerebral Infarction in Sickle Cell Anemia - Comprehensive Sickle Cell Center
Conditions: Anemia, Sickle Cell; Blood Disease; Cerebrovascular Disorders; Cerebrovascular Accident

21. **Completed** A Phase I/II Trial of Recombinant-Methionyl Human Stem Cell Factor (SCF) in Adult Patients with Sickling Disorders

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Conditions: hemoglobin SC disease; sickle cell anemia

22. Recruiting — Home Based Massage and Relaxation for Sickle Cell Pain
Condition: Sickle Cell Disease

23. Recruiting — A Stratified Sickle Event Randomized Trial (ASSERT)
Condition: Sickle Cell Disease

24. Recruiting — Study of Clotrimazole and Hydroxyurea in Patients With Sickle Cell Syndromes
Condition: Sickle Cell Anemia

25. No longer recruiting — Phase III Randomized Study of Poloxamer 188 for Vaso-Occlusive Crisis of Sickle Cell Disease
Condition: Sickle Cell Anemia

26. Recruiting — Phase II Randomized Trial of Arginine Butyrate Plus Standard Local Therapy in Patients With Refractory Sickle Cell Ulcers
Conditions: Skin Ulcers; Sickle Cell Anemia

27. Recruiting — Phase I/II Randomized Study of Hydroxyurea With or Without Clotrimazole in Patients With Sickle Cell Anemia
Condition: Sickle Cell Anemia

28. Completed — Pilot Study of Fructose for Sickle Cell Crisis
Condition: Sickle Cell Anemia

29. Completed — Investigation of Selected Patient Groups From The Cooperative Study of Sickle Cell Disease
Conditions: Anemia, Sickle Cell; Blood Disease

30. Completed — Neuropsychological Studies of Children with Sickle Cell
Conditions: Blood Disease; Anemia, Sickle Cell; Neurologic Manifestations

31. Recruiting — Stem Cell Transplantation after Reduced-Dose Chemotherapy for Patients with Sickle Cell Disease or Thalassemia
Conditions: Hemoglobinopathies; Anemia, Sickle Cell; Hemoglobin SC Disease; Thalassemia; Thalassemia Major

32. Recruiting — Induction of Stable Chimerism for Sickle Cell Anemia
Conditions: Blood Disease; Hematopoietic Stem Cell Transplantation; Anemia, Sickle Cell

33. Recruiting — L-glutamine Therapy for Sickle Cell Anemia
Conditions: Anemia, Sickle Cell; Thalassemia

34. Completed — Penicillin Prophylaxis in Sickle Cell Disease (PROPS)
Conditions: Anemia, Sickle Cell; Hematologic Diseases; Hemoglobinopathies; Infection (S. pneumoniae); Pneumonia

35. No longer recruiting — Multicenter Study of Hydroxyurea in Patients With Sickle Cell Anemia (MSH)
Conditions: Anemia, Sickle Cell; Hematologic Diseases; Hemoglobinopathies

36. Completed — Stroke Prevention in Sickle Cell Anemia (STOP 1)
Conditions: Anemia, Sickle Cell; Cerebral Embolism and Thrombosis; Cerebrovascular Disorders; Hematologic

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Diseases; Hemoglobinopathies

37. **Completed** Pediatric Hydroxyurea in Sickle Cell Anemia (PED HUG)
Conditions: Anemia, Sickle Cell; Hematologic Diseases; Hemoglobinopathies

38. **Completed** Cooperative Study of The Clinical Course of Sickle Cell Disease
Conditions: Anemia, Sickle Cell; Blood Disease

39. **Completed** Indices of Severity and Prognosis for Sickle Cell Disease
Conditions: Anemia, Sickle Cell; Blood Disease

40. **Completed** Hemostasis in Sickle Cell Disease--Infancy to Adulthood
Conditions: Anemia, Sickle Cell; Blood Disease

41. **Recruiting** Pediatric Hydroxyurea in Sickle Cell Anemia (BABY HUG)
Conditions: Blood Disease; Anemia, Sickle Cell

42. **No longer recruiting** Pain in Sickle Cell Epidemiologic Study
Conditions: Blood Disease; Anemia, Sickle Cell

43. **No longer recruiting** Pediatrics:Chlamydia, Sickle Cell Anemia and Stroke Risk - Ancillary to STOP II
Conditions: Blood Disease; Anemia, Sickle Cell; Chlamydia Infections; Cerebrovascular Accident

44. **Recruiting** Silent Cerebral Infarct Multi-Center Clinical Trial
Conditions: Sickle Cell Anemia; Stroke

45. **No longer recruiting** Stroke Prevention in Sickle Cell Anemia (STOP 2)
Conditions: Blood Disease; Cerebrovascular Accident; Anemia, Sickle Cell

46. **Completed** Study of Allogeneic Bone Marrow Transplantation Using Matched, Related Donors in Patients With Nonmalignant Hematologic Disorders
Conditions: Neutropenia; Sickle Cell Anemia; Thalassemia Major; Red-Cell Aplasia, Pure

47. **Recruiting** Phase I/II Study of Nonmyeloablative Allogeneic Bone Marrow Transplantation in Patients With High Risk Hemoglobinopathy
Conditions: Graft Versus Host Disease; Sickle Cell Anemia; Thalassemia

48. **No longer recruiting** Transcranial Doppler (TCD) Ultrasound of Subjects Enrolled in BABY HUG - Ancillary to BABY HUG
Conditions: Blood Disease; Anemia, Sickle Cell; Cerebrovascular Disorders; Cerebral Embolism and Thrombosis

49. **Terminated** Bone marrow transplant from donor using less toxic conditioning for patient with high risk hemoglobinopathies
Conditions: Sickle Cell Anemia; Hemoglobinopathy; Thalassemia

50. **Terminated** Bone Marrow transplant from related donor for patients with high risk hemoglobinopathies

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	Conditions: Sickle Cell Anemia; Hemoglobinopathy; Thalassemia
51. Recruiting	Improving the Results of Bone Marrow Transplantation for Patients with Severe Congenital Anemias Conditions: Congenital Hemolytic Anemia; Diamond-Blackfan Anemia
52. Not yet recruiting	Sibling Donor Cord Blood Banking and Transplantation Conditions: Blood Disease; Anemia, Sickle Cell; Anemia (Cooley's); Leukemia; Bone Marrow Transplantation; Hematopoietic Stem Cell Transplantation
53. Recruiting	Development of a Hospital-Based Home Program for the Use of Inhaled Nitric Oxide in the Chronic Management of Severe Cardiopulmonary Diseases Conditions: Pulmonary Hypertension; Lung Disease; Sickle Cell Disease; Cardiac transplant; Lung transplant
54. Recruiting	The Effect of Oral Magnesium Pidolate on How Often Painful Crises Happens in Patients with Hemoglobin SC Disease Condition: Hemoglobin SC Disease
55. No longer recruiting	Surgery With or Without Chemotherapy in Treating Patients With Stage I Non-small Cell Lung Cancer Condition: stage I non-small cell lung cancer

[0006] It is seen that numerous of these studies concern the use of hydroxyurea (either alone or in combination with another drug), bone marrow transplantation, nitric oxide, or clotrimazole. A small number of the listed studies are testing other drugs, e.g., hydroxyurea plus L-arginine or sildenafil; nitric oxide plus nitroglycerin; ICA-17043; ICL60 (Deferasirox) as compared to deferoxamine; poloxamer 188; atorvastatin; arginine; L-glutamine; arginine butyrate; fructose; and oral magnesium pidolate.

[0007] In addition to the studies listed above, numerous articles have been published concerning the use of other drugs to treat sickle cell patients. These drugs work in a large variety of ways. Abdulmalik et al. (Br. J. Haematol. 128:552-561 (2005)) used 5-hydroxymethyl-2-furfural which binds to intracellular sickle hemoglobin and inhibits sickling. Vanillin is known to bind to sickle hemoglobin and inhibit sickling, but given orally it is rapidly decomposed. Zhang et al. (Br. J. Haematol. 125:788-795 (2004)) used a vanillin prodrug, MX-1520, which is not rapidly decomposed and found that it reduced the percentage of sickled cells in the blood. DeBellis et al. (Blood Cells Mol. Dis. 31:286-290 (2003)) tested three

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purine-based antiviral agents (ganciclovir, valacyclovir, and penciclovir) and found that valacyclovir and ganciclovir show anti-sickling activity similar to acyclovir which was known to inhibit aggregation of hemoglobin S and the sickling of erythrocytes. Cordeiro and Oniyangi (Cochrane Database Syst. Rev. 3:CD004448 (2004)) found that a phytomedicine (Niprisan) was effective in reducing episodes of sickle cell disease crisis associated with severe pain. Iyamu et al. (Br. J. Haematol. 122:1001-1008 (2003)) also studied Niprisan (Nix-0699) in mice and found that it inhibited sickling. Manion et al. (Clin. Pharmacol. Ther. 69:346-355 (2001)) studied the use of aspartame for treating sickle cell disease. Aspartame binds to two human Bence Jones proteins (Mcg and Sea). In vivo studies in persons showed that aspartame treatment led to decreased sickling. Beddell et al. (Br. J. Pharmacol. 82:397-407 (1984)) designed substituted benzaldehydes to bind preferentially to the oxy conformation of human hemoglobin to stabilize the oxygenated form of hemoglobin and thereby increase its oxygen affinity. At least one such compound was a potent inhibitor at low oxygen pressure of the sickling of erythrocytes. Related work by Arya et al. (Br. J. Haematol. 93:817-821 (1996)) involved the study of Tucaresol (589C80;4[2-formyl-3-hydroxyphenoxymethyl]benzoic acid) which is a substituted benzaldehyde. Administration of tucaresol to sickle cell patients resulted in decreased hemolysis, a decrease in lactate dehydrogenase, and a halving of the irreversibly sickled cell counts. However, 3 of 6 patients developed fever and cervical lymphadenopathy. Riddington and De Franceschi (Cochrane Database Syst. Rev. 4:CD003426 (2002)) reviewed the literature of controlled trials to find trials related to using drugs which aimed to prevent sickle cell related crises by reducing red blood cell dehydration. They found two trials. These tested zinc sulfate and piracetam. The zinc trial showed a significant reduction in the total number of pain, hemolytic, aplastic and sequestration crises over one and a half years. The piracetam trial showed a reduction in pain crises over a one year time span, although blood counts were not significantly changed. Dehydration of red blood cells can occur through the Ca-activated K channel which depends on the parallel movement of Cl ions. Bennekou et al. (Blood 97:1451-1457 (2001)) studied whether Cl-conductance block might prevent dehydration of sickle red blood cells. They used a novel Cl-conductance inhibitor (NS3623). Mice treated with NS3623

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showed an increase in hematocrit, a decrease in the mean corpuscular hemoglobin concentration, and a loss of the densest red cell population with a shift from a high proportion of sickled to well-hydrated discoid erythrocytes. Kocak et al. (Br. J. Haematol. 92:329-331 (1996)) used the vasodilating Ca^{+2} channel blocker bencyclane in 18 patients with sickle cell disease. The patients' blood contained fewer irreversibly sickled cells. Inhibition of the sickling-induced fluxes of Na^{+}, K^{+} and Ca^{+2} was studied by Joiner et al. using dipyridamole (Blood 97:3976-3983 (2001)). The dipyridamole was found to inhibit sickling by inhibiting the ion flux. Shartava et al. (Am. J. Hematol. 62:19-24 (1999)) studied both clotrimazole (which is a specific inhibitor of the Ca^{+2} activated potassium channel) and the antioxidant N-acetylcysteine. Both were found to inhibit the in vitro formation of high-density sickle cells. The authors concluded that N-acetylcysteine protects the potassium channel from oxidative damage caused by diminished levels of reduced glutathione. Another drug which protects against K^{+} efflux is bepridil and this was found to significantly inhibit the formation of irreversibly sickled cells in a study by Johnston et al. (Br. J. Haematol. 73:522-526 (1989)). A recent review of established and experimental treatments for sickle cell disease was published by De Franceschi and Corrocher (Haematologica 89:348-356 (2004)), the contents of which are incorporated herein by reference.

[0008] In addition to the numerous studies listed above, basic research has been performed which shows a possible relationship between sickle cell disease and complement. The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

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[0009] 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/or destruction of target cells.

[0010] 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. Complement activation can also occur via the lectin complement pathway (LCP). Lectins are carbohydrate-binding proteins that recognize oligosaccharide structures present on cell surfaces, the extracellular matrix, and secreted glycoproteins. As with the classical complement pathway and the alternative pathway, the LCP also converges at the point of cleavage of complement component C3. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

[0011] C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon (1983). In Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals).

[0012] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., Complement Inflamm. 8:328-340 (1991)). This property of C3b is regulated

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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.

[0013] C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., J. Immunol. 146:362-368 (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.

[0014] 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.

[0015] 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, J. Immunol. 119:1597-1602 (1977); Wetsel and Kolb, J. Immunol. 128:2209-2216 (1982)) and acid treatment (Yamamoto and Gewurz, J. Immunol. 120:2008-2015 (1978); Vogt et al., Molec. Immunol. 26:1133-1142 (1989)) can also cleave C5 and produce active C5b.

[0016] 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 and polymerization 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)

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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.

[0017] In 1994 Chudwin et al. (Clin. Immunol. Immunopathol. 71:199-202 (1994)) reported that red blood cells from sickle cell patients activated complement to a greater extent than did red blood cells from control patients. Additionally they reported that the activation was via the alternative pathway and not the classical pathway. In 1995 Mold et al. (Clin. Immunol. Immunopathol. 76:314-320 (1995)) demonstrated that the alternative pathway activation is initiated by membrane phospholipid changes which occur in sickled erythrocytes. They found that in patients with intermittent pain there was little evidence of complement activation and that there was an increased plasma concentration of C3a during a painful crisis. Also, elevated C3a and C4d levels were observed in patients with continuous pain. Earlier, Tomasko and Chudwin had shown that changes in phospholipid organization in sickle cells may contribute to the chronic alternative complement pathway activation seen in sickle cell disease patients (J. Lab. Clin. Med. 112:248-253 (1988)). In 1986 De Ceulaer et al. (J. Clin. Lab. Immunol. 21:37-41 (1986)) published a paper concerning complement levels in sickle cell disease patients and the relation to infection. They reported that complement component C3 levels were consistently lower in sickle cell children and levels of C3d were significantly higher, but they found no difference in the prevalence of infections between the groups. Dieye et al. (Dakar Med. 44:175-179 (1999)) also studied the relationship between complement and infection in sickle cell patients. They found that C3c complement (indicative of the alternative pathway) levels were decreased significantly whereas

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C4c (indicative of the classical pathway) levels were not. They conclude there is a direct involvement of the complement system in sickle cell disease and the depletion of C3 is a possible cause of increased susceptibility to infections in patients with sickle cell disease.

[0018] Mohamed et al. (Am. J. Trop. Med. Hyg. 49:799-803 (1993)) found that C3d levels are elevated in sickle cell disease patients. They noted that this was not accompanied by altered complement activity in serum in vitro or in C3 reduction. They state that the underlying mechanism of this activation may be the triggering of the complement reaction by the sickled cell, the phospholipid organization of which appears to promote the alternative pathway complement activation. They conclude that the complement activation may contribute to impaired host defense against bacterial infections.

[0019] Test and Woolworth (Blood 83:842-852 (1994)) further studied the relationship between sickle cell disease and complement. They first noted that prior work concluded that intravascular hemolysis of sickle cells resulted in complement activation. Test and Woolworth took the opposite view that possibly complement activation was leading to hemolysis. They showed in a series of in vitro assays that regulation of complement in sickle cells was abnormal, especially in that increased binding of C5b-7 and C9 to denser sickle cells was seen and this appeared to increase the susceptibility of sickle cells to lysis. They believe that the increased binding is most likely caused by changes in MIRL (also referred to as CD59) brought about by the sickling process and which is a membrane protein which inhibits lysis by the membrane attack complex. CD59 is a human glycosylphosphatidylinositol-anchored membrane glycoprotein that serves as the principle inhibitor of the cytolytic and pore forming activities of the C5b-9 membrane attack complex (MAC) of human complement. By inhibiting MAC, CD59 protects human blood cells, vascular endothelium and other cells from complement, which is normally present in human plasma and other body fluids. Although the exact mechanism of its complement inhibitory function remains to be elucidated, CD59 is known to bind peptide segments of C8 and C9 that become exposed when these complement proteins incorporate into the C5b-9 complex, thereby preventing initiation or propagation of the membrane-inserted C9 polymer

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that is responsible for membrane damage. Blood cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) lack the ability to anchor CD59 in their membranes and these cells are very susceptible to lysis via complement. Transducing these cells with a recombinant transmembrane form of CD59 results in the surface expression of CD59 and these transduced cells are protected against classical complement-mediated membrane damage by human serum (Rother et al., Blood 84:2604-2611 (1994)).

[0020] This work was followed up by Liu et al. (Blood 93:2297-2301 (1999)) who inserted various types of lipids into human erythrocyte membranes. They found that neutral lipids had no effect on C5b-7 uptake or hemolysis by C5b-9, but acidic phospholipids did cause a dose-dependent increase in both lysis and C5b-7 uptake. They concluded that the presence of anionic lipids on the exterior of the membrane increases C5b-7 uptake and subsequent hemolysis. They point out that it is known that sickle cell erythrocytes have increased exposure of phophatidylserine on their external face and are abnormally sensitive to lysis by C5b-9.

[0021] Concerning the issue of hemolysis, it is known that in autoimmune hemolytic anemia red blood cell destruction is primarily extravascular. This is largely mediated through either Fc and/or complement receptors expressed by phagocytic effector cells. Experimentally induced autoimmune hemolytic anemia occurs even in the absence of the complement components C3, C4 and C5 but requires the presence of Fc receptors in association with the common FcR γ-chain (Meyer et al., Blood 92:3997-4002 (1998)).

[0022] The prior art as a whole leaves the field quite uncertain as to what role, if any, complement plays in sickle cell disease and more specifically complement's role in lysis of red blood cells in sickle cell disease patients. Mohamed et al. (Am. J. Trop. Med. Hyg. 49:799-803 (1993)) state, "Although some investigators have reported defects in the complement system as the cause for the increased susceptibility to infections, others have not found such defects." They also state that intravascular hemolysis accounts for the destruction of one-third of the sickled erythrocytes, while two-thirds are removed by macrophages in the spleen and other locations and that the hemolytic process in sickle cell anemia may involve several components, including activation of complement, macrophages, and neutrophils.

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[0023] The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are incorporated herein by reference.

SUMMARY

[0024] Methods for treating patients suffering from sickle cell disease are presented. A method of treating sickle cell disease in a mammal comprising treating said mammal with a therapeutically effective amount of an anti-C5 antibody, wherein said treatment results in decreased lysis of red blood cells in said mammal. In certain embodiments, said mammal is a human.

[0025] In certain embodiments, said antibody is a whole antibody or an antibody fragment. In certain embodiments, said whole antibody or antibody fragment is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', or an $F(ab')_2$.

[0026] In certain embodiments, said treatment is chronic. In certain embodiments, said treatment comprises treating an acute episode. In certain embodiments, said mammal is treated by administering said antibody intravenously.

[0027] In certain embodiments, said mammal is treated to inhibit or decrease the amount and/or extent of an undesirable physiological condition resulting from excess lysis of red blood cells. In certain embodiments, said undesirable physiological condition is selected from the group consisting of dystonia, a clotting disorder, pulmonary hypertension, systemic hypertension, gastrointestinal contractions, abdominal pain, sternal pain, erectile dysfunction, priapism, inflammation, esophageal spasm, dysphagia, thrombosis, decreased organ perfusion, platelet activation and death.

[0028] In certain embodiments, said antibody inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway. In certain embodiments, said antibody is an antibody against a member of the complement cascade components such as for example, C5, C5b, C6, C7, C8, and C9. In certain

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embodiments, said antibody is an inhibitor of C5 cleavage. In certain embodiments, said antibody decreases the availability of downstream complement components.

[0029] In certain embodiments, said antibody is pexelizumab. In certain embodiments, said antibody is eculizumab.

DETAILED DESCRIPTION

[0030] Sickle cell disease is an extremely serious disease affecting approximately 72,000 Americans and many more persons in other countries. At present a cure is almost nonexistent, the only cure being a bone marrow transplant. Gene therapy is a possible cure but is not being performed. In general sickle cell disease patients have their symptoms treated but are not cured. Treatments include treating or preventing infections by administration of antibiotics, blood transfusions to increase the supply of normal red blood cells, drugs to increase the level of fetal hemoglobin which is able to carry oxygen, drugs to prevent loss of water from erythrocytes thereby helping to prevent sickling, and drugs to aid in keeping blood vessels open. Despite these treatments, many patients continue to suffer greatly and many patients die as a result of the disease. More effective treatments are needed.

[0031] Several studies dating back several years have shown that complement activation may be affected in sickle cell disease patients. These date back to at least 1967 (Francis and Womack, Am. J. Med. Technol. 33:77-86 (1967)). However the role of complement activation is still unclear. There are conflicting reports as to whether changes in complement result in greater infection. Even if it is proved that infection is greater because of effects to the complement system, such infections are normally treated with inexpensive antibiotics such as penicillin. Other work shows conflicting results as to whether sickle cell disease causes complement activation or whether complement activation causes some of the problems of sickle cell disease.

[0032] Applicant here proposes that treatment of a sickle cell disease patient with a complement inhibitor will decrease lysis of red blood cells and at least some of the symptoms seen in sickle cell patients. Many of the symptoms are a direct result of the lysis of red blood cells. One aspect of the lysis of red blood cells is the consequent excess free hemoglobin in the bloodstream. This can lead to smooth muscle dystonias involving the gastrointestinal, cardiovascular, pulmonary, and

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urogenital systems, as well as clotting disorders, these symptoms including gastrointestinal contractions, erectile dysfunction such as priapism, and pulmonary hypertension as well as to clot formation (Rother et al., JAMA 293:1653-1662 (2005), the contents of which are specifically incorporated herein by reference). Approximately one-third of sickled red blood cells are lysed via intravascular hemolysis (Mohamed et al., Am. J. Trop. Med. Hyg. 49:799-803 (1993)). Applicant here proposes that inhibition of complement activation will decrease the amount of lysis and thereby decrease the amount and/or extent of symptoms related to the lysis of red blood cells in sickle cell disease patients. Inhibitors of members of the complement cascade including for example antibodies to components such as C5, C5b, C6, C7, C8, and C9. In particular embodiments, this application contemplates the use of the anti-C5 antibody eculizumab (a whole antibody) which is known to inhibit cleavage of C5 into C5a and C5b. Treatment of patients with such inhibitors thereby decreases complement mediated lysis of red blood cells. Long lasting inhibitors are preferable. Eculizumab, being a whole antibody, is longer lasting in vivo than is a single-chain antibody and is preferably used although shorter acting inhibitors may be used, especially if used in time of a crisis and is used to relieve the crisis. Eculizumab has been used in clinical studies and found to be well tolerated with minimal side effects. Eculizumab has been administered to patients with paroxysmal nocturnal hemoglobinuria which is a disease in which red blood cells are easily lysed due to a lack of a protein in the red blood cell membrane which protects against lysis by activated complement. Most patients are administered a dose once every two weeks. See Hillmen et al., New Engl. J. Med. 350:552-559 (2004), the contents of which are specifically incorporated herein by reference.

[0033] 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 down stream complement components.

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[0034] Additional proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0035] 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.

[0036] 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

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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.

[0037] 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.

[0038] 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.

[0039] "Fv" usually refers to the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although likely at a lower affinity than the entire binding site.

[0040] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

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[0041] This disclosure also provides monoclonal anti-C5 antibodies. A monoclonal antibody can be obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., Nature 256:495-497 (1975), or may be made by recombinant DNA methods (*see, e.g.*, U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature 352:624-628 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example.

[0042] General methods for the immunization of animals (in this case with C5 and/or C5b, etc.), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal 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

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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).

[0043] 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.

[0044] 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 mixture of antibodies that are applicable for an intended purpose and desired need.

[0045] 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,

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such as a human antibody, or from a humanized antibody. Human or humanized antibodies are specific for administration to human patients.

[0046] "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.

[0047] SMIPs are a class of single-chain peptide engineered to include a target binding region, effector domain (CH2 and CH3 domains). *See, e.g.*, U.S. Patent Application Publication No. 20050238646. The target binding region may be derived from the variable region or CDRs of an antibody, e.g., an anti-C5 antibody of the invention. Alternatively, the target binding region is derived from a protein that binds C5.

[0048] 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).

[0049] An "isolated" antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In specific embodiments, the antibody will be purified to greater than 95% by weight of antibody as determined by the Lowry method, or greater than 99% by weight, to a degree that complies with applicable regulatory requirements for administration to human patients (e.g., substantially pyrogen-free), to a degree sufficient to obtain at

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least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells, since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step, for example, an affinity chromatography step, an ion (anion or cation) exchange chromatography step, or a hydrophobic interaction chromatography step.

[0050] 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)).

[0051] 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.

[0052] In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced.

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Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen-binding function of a corresponding full-length antibody (such as for example, ability of anti-C5 antibody to bind C5).

Pharmaceutical Formulations and Uses

[0053] Methods of administration of antibodies are well-known to those of skill in the art. To achieve the desired inhibition, the antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood. The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/mL to about 500 μg/mL. However, greater amounts may be required for extreme cases and smaller amounts may be sufficient for milder cases.

[0054] Administration of the 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

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auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like. 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 sickle cell treatments known in the art (supra).

INCORPORATION BY REFERENCE

[0055] All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

[0056] The present methods are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLE

PROPHYLACTIC TREATMENT OF A

SICKLE CELL DISEASE PATIENT WITH ECULIZUMAB

[0057] Sickle cell disease patients have episodes of hemolysis in which large amounts of red blood cells are lysed and hemoglobin is released into the plasma. Because of the recurring nature of this disease, such patients will be treated prophylactically by administering anti-C5 antibodies at regular intervals such that active antibody is present in the plasma at any time and can bind to C5 and inhibit its activation, thereby decreasing the amount of lysis of red blood cells. The amount of antibody required will depend upon the severity of the disease. Antibodies are degraded over time and must be replaced. As an example to be used as guidance, PNH patients are administered an anti-C5 antibody at a dose of 900 mg

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approximately once every two weeks to maintain an active level of that antibody (Hillmen et al., N. Engl. J. Med. 350:552-559 (2004)) to decrease lysis of red blood cells. The amount delivered depends upon the form of the antibody and its *in vivo* half-life.

[0058] While the invention has been disclosed by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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CLAIMS:

1. A method of treating sickle cell disease in a mammal comprising treating said mammal with a therapeutically effective amount of an anti-C5 antibody, wherein said treatment results in decreased lysis of red blood cells in said mammal.

2. The method of claim 1 wherein said mammal is a human.

3. The method of claim 1 wherein said antibody is a whole antibody or an antibody fragment.

4. The method of claim 3 wherein said whole antibody or antibody fragment is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', or an $F(ab')_2$.

5. The method of claim 1 wherein said treatment is chronic.

6. The method of claim 1 wherein said treatment comprises treating an acute episode.

7. The method of claim 1 wherein said mammal is treated by administering said antibody intravenously.

8. The method of claim 1 wherein said mammal is treated to inhibit or decrease the amount and/or extent of an undesirable physiological condition resulting from excess lysis of red blood cells.

9. The method of claim 8 wherein said undesirable physiological condition is selected from the group consisting of dystonia, a clotting disorder, pulmonary hypertension, systemic hypertension, gastrointestinal contractions, abdominal pain, sternal pain, erectile dysfunction, priapism,

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inflammation, esophageal spasm, dysphagia, thrombosis, decreased organ perfusion, and death.

10. The method of claim 1 wherein said antibody inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway.

11. The method of claim 1 wherein said antibody is an antibody against any member of the group consisting of the complement components C5, C5b, C6, C7, C8, and C9.

12. The method of claim 1 wherein said antibody is an inhibitor of C5 cleavage.

13. The method of claim 1 wherein said antibody is pexelizumab.

14. The method of claim 1 wherein said antibody is eculizumab.

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(71) Applicant *(for all designated States except US)*: **MEDIMMUNE, INC.** [US/US]; One MedImmune Way, Gaithersburg, MD 20878 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ALLAN, Christian** [US/US]; 4401 Gregg Road, Brookeville, MD 20833 (US). **WU, Herren** [US/US]; 14405 Harvest Moon Road, Boyds, MD 20841 (US). **SWERS, Jeffery** [US/US]; 310 Market St. West #335, Gaithersburg, MD 20878 (US). **DALL'ACQUA, William** [FR/US]; 564 Chesterstown Street, Gaithersburg, MD 20878 (US).

(74) Agents: **KREPPEL, Lisa** et al.; Medimmune, INC., One MedImmune Way, Gaithersburg, MD 20878 (US).

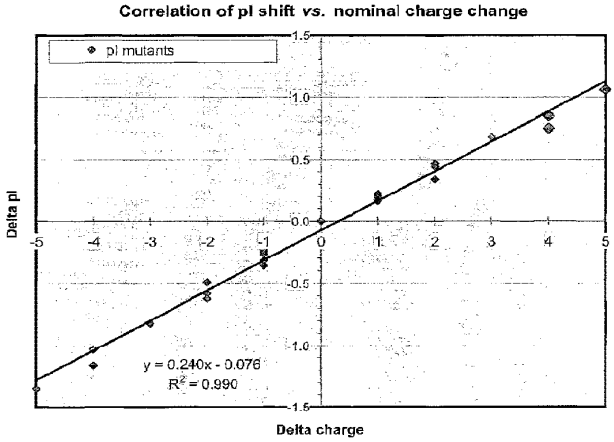
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(54) **Title:** AN INTEGRATED APPROACH FOR GENERATING MULTIDOMAIN PROTEIN THERAPEUTICS



(57) **Abstract:** The invention provides method for therapeutic protein drug development that incorporates therapeutic and/or formulation and/or manufacturing considerations in the early screening process. The approach involves screening a plurality of different variants of a domain that have been determined to have the desired therapeutic property to identify one or more variants that have desired therapeutic and/or formulation characteristics, and constructing the full multidomain proteins using the identified domain variants. The present invention also provides a method for determining the shelf life of multidomain proteins in formulations. The method comprises determining a thermal denaturation and/or renaturation curve of a domain of the protein whose unfolding leads to aggregation of the protein in a solution. The method evaluates the shelf life of the multidomain protein based on the denaturation/renaturation curve. The invention also provides methods for engineering multidomain proteins to improve their therapeutic and/or formulation characteristics.

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AN INTEGRATED APPROACH FOR GENERATING MULTIDOMAIN PROTEIN THERAPEUTICS

1. FIELD OF THE INVENTION

[0001] The invention relates to a method for therapeutic protein development that allows generation of proteins of both high therapeutic efficacy and optimal formulation characteristics, *e.g.*, high solubility and long shelf life. The present invention also relates to a method for evaluating one or more metrics, which provide an indication of the shelf life of a multidomain protein formulation.

2. BACKGROUND OF THE INVENTION

[0002] A wide variety of biologically active proteins and polypeptides can now be produced in sufficiently large quantities for use as drugs. For example, the development of the hybridoma method and recombinant DNA techniques have made it possible to produce antibodies on large scale. This has allowed wide use of pharmaceutical compositions containing proteins, such as antibodies, for treating a variety of diseases. Such treatments normally require administering to a patient the proteins at high concentrations.

[0003] However, a protein that has desired therapeutic properties may not have sufficiently high solubility. Even for those proteins that have high solubility, high concentration liquid formulations tend to have short shelf lives and may lose biological activity as a result of chemical and physical instabilities during the storage. Additionally, proteins are generally more viscous at high concentrations, which can complicate packaging, storage and delivery of the protein therapeutic. Furthermore, chemical instability may be caused by deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange, and physical instability may be caused by protein denaturation, aggregation, precipitation or adsorption. Among those, aggregation, deamidation and oxidation are known to be the most common causes of antibody degradation (Wang *et al.*, 1988, *J. of Parenteral Science & Technology* 42 (Suppl.):S4-S26; Cleland *et al.*, 1993, *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4):307-377; Manning *et al.*, 1989, Pharm. Res. 6:903-918). Aggregate formation during storage of a liquid protein composition can adversely affect the biological activity of the protein, resulting in loss of therapeutic efficacy and/or an increase in immunogenicity in humans. Aggregate formation may also cause other problems

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such as blockage of tubing, membranes, or pumps when the protein composition is administered using an infusion system.

[0004] Due to the instability of proteins in liquid pharmaceutical formulations, protein therapeutics are often packaged in the lyophilized form along with a suitable liquid medium for reconstitution. Although lyophilization improves storage stability of the composition, many proteins exhibit decreased activity, either due to storage in the dried state (Pikal, 1990, Biopharm. 27:26-30) or as a result of aggregate formation or loss of catalytic activity upon reconstitution as a liquid formulation (see, for example, Carpenter *et al.*, 1991, Develop. Biol. Standard 74:225-239; Broadhead *et al.*, 1992, Drug Devel. Ind. Pharm. 18:1169-1206; Mumenthaler *et al.*, 1994, Pharm. Res. 11:12-20; Carpenter *et al.*, 1988, Cryobiology 25:459-470; and Roser, 1991, Biopharm. 4:47-53). Lyophilized formulations of antibodies also require a prolonged process for lyophilization and high cost for manufacturing. A lyophilized formulation has to be reconstituted aseptically and accurately by healthcare practitioners prior to administering to patients. The reconstitution procedure is cumbersome and the time limitation after the reconstitution can cause great inconveniences in administering the formulation to patients, leading to significant waste, if not reconstituted properly or if the reconstituted dose is not used within six (6) hours and must be discarded. Reconstitution may also increase the possibility of incorrect dosing. Thus, a lyophilized formulation which is more stable and is readily reconstituted with little loss in potency is desirable.

[0005] A desirable alternative to lyophilized formulations is liquid formulations of protein therapeutics having concentrations comparable to or higher than the reconstituted lyophilized formulations. Such liquid formulations of protein therapeutics can be administered to a subject without the need of reconstitution, thereby allowing healthcare practitioners much quicker and easier administration of protein therapeutics to a patient. In addition, the manufacturing process of the liquid formulation protein therapeutics is simpler and more efficient than the manufacturing process for the lyophilized version because all stages of the manufacturing of the liquid formulations are carried out in an aqueous solution, involving no drying process, such as lyophilization. Accordingly, it is also more cost effective. The development of high concentration, ready-to-use, liquid formulations of protein therapeutics has thus attracted great attention in the biopharmaceutical industry.

[0006] Stability of proteins and polypeptides in pharmaceutical formulations, both liquid and lyophilized, can be affected, for example, by factors such as pH, ionic strength,

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temperature, repeated cycles of freeze-thaw, and exposure to mechanical shear forces such as occur during processing. Various highly stable, high concentration liquid formulations have been successfully developed. For example, liquid formulations of antibodies that are stable for more than 5 years when stored at 4°C have been reported. U.S. Patent No. 6,525,102 discloses a stabilized liquid polypeptide-containing pharmaceutical composition. The composition comprises an amino acid base, which serves as the primary stabilizing agent of the polypeptide, and an acid and/or its salt form to buffer the solution within an acceptable pH range for stability of the polypeptide. The compositions are near isotonic. The `102 patent also discloses methods for increasing stability of a polypeptide in a liquid pharmaceutical composition and for increasing storage stability of such a pharmaceutical composition. Lyophilized formulations are common and their stability and reconstitution characteristics may be modified by the addition of stabilizers and/or excipients. However, the development of such liquid and lyophilized formulations depends on the particular protein therapeutics, and often requires significant optimization efforts. Thus, improving the stability of pharmaceutical compositions containing protein therapeutics of a pharmaceutically effective concentration remains a challenge. An additional challenge is providing for formulations which have low enough viscosity to be readily manufactured and/or administered at high concentrations.

[0007] In a traditional approach to therapeutic protein (*e.g.*, antibody) development, the protein that has a desired activity and/or property (*e.g.*, binding affinity) is first generated. The protein is then submitted for formulation development to determine the optimal formulation and storage conditions. Traditional screening and optimization processes require lengthy stability studies which are both time consuming and can only examine a limited number of potential formulations. If the protein should fail to meet the formulation requirements, the whole drug development process essentially fails. Thus, there is a need for methods that allow incorporating formulation considerations into early stages of the drug development process. In addition, since the desired shelf life can be as long as one year or longer, methods relying monitoring the formulations in real time are inefficient. Attempts have been made to develop methods for rapidly screening formulations. For example, U.S. Patent No. 6,232,085 discloses a multi-variable method for optimizing the shelf life of a protein which is capable of denaturing due to a thermal change. The method comprises contacting the target molecule with one or more of a multiplicity of different molecules or different biochemical conditions in each of a multiplicity of containers, simultaneously

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heating the multiplicity of containers, measuring in each of the containers a physical change associated with the thermal denaturation of the target molecule resulting from heating, generating a thermal denaturation curve for the target molecule as a function of temperature for each of the containers, comparing each of the denaturation curves to (i) each of the other thermal denaturation curves and to (ii) the thermal denaturation curve obtained for the target under a reference set of biochemical conditions, and ranking the efficacies of multiplicity of different molecules or the different biochemical conditions according to the change in each of the thermal denaturation curves. However, such methods are cumbersome and do not address the address the intrinsic properties or characteristics, such as, pI and Tm, of the protein to be formulated.

[0008] The intrinsic properties of proteins not only affect their formulation characteristics but may have serious implications for their therapeutic use as well. For example, studies have shown that recombinant toxins made up of a cell-targeting Fv portion of an antibody fused to a toxin have non-specific dose limiting toxicities (*e.g.*, non-specific liver toxicities) which are attributable to the high isoelectric point (pI) of the Fv portion of the molecule. Lowering the pI of the Fv portion of these recombinant toxins by site directed mutagenesis reduced their non-specific toxicity in animal models without altering reducing their antitumor activity (Onda *et al.*, 1999, J. Immunol., 163: 6072-77, Onda *et al.*, 2001, Cancer Res., 61: 5070-77). Likewise, lowering the pI of a radiolabelled anti-tumor dsFv (disulfide stabilized Fv) by chemical modification increased renal clearance thereby decreasing the buildup of radioactivity in the kidney (Kim *et al.*, 2002, Nucl. Med. Biol., 29: 795-801).

[0009] In other studies the transendothelial migration and endocytosis of antibodies was enhanced by cationization to increase the pI. The cationized antibodies retained their binding affinity and were rapidly internalized into cells with minimal non-specific toxicity or immunogenicity (Pardrige *et al.*, 1998, J. Pharmaol. Exp. Therap., 286: 548-54). Cationization of antibodies has also been shown to enhance the delivery of antibodies across the blood brain barrier (Triguero *et al.*, 1989, PNAS, 86: 4761-4765) These data indicate that there may be an optimal pI for certain therapeutic proteins, such as antibodies or chimeric proteins made up of antibody domains. Particularly those proteins which carry a toxin, are required in large doses for optimal therapeutic response or those which are required intracellularly or in extravascular compartments.

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[0010] Thus, there is a need for more efficient methods that allow quick indication of the shelf life and/or clinical properties of protein formulations based on the intrinsic properties of the protein. Additionally, because certain intrinsic properties, such as pI and Tm, are generally not selection criterion when therapeutic proteins (*e.g.*, antibodies) are developed, proteins which are therapeutically active may have suboptimal formulation or clinical properties. Methods to engineer certain intrinsic properties, such as pI and Tm, downstream of, or concurrently with development would allow the rapid production of proteins with preferred formulation and therapeutic characteristics.

[0011] Discussion or citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

[0012] The invention is based in part on the discovery of the inventors that certain therapeutic and/or formulation and/or manufacturing characteristics of a multidomain protein can be evaluated by examining individual domains that form the protein. One or more metrics representing the physical and/or chemical and/or structural characteristics of protein domains are determined and individual domains are then selected based on their metrics. The individual domains may be examined in the context of the intact multidomain protein or as isolated domains. For example, the physical/chemical (including structural) characteristics of a Fab or an Fc domain in an antibody may determine the biodistribution and/or non-specific toxicity and/or stability and/or solubility and/or viscosity of the antibody. An approach incorporating selecting appropriate domains having optimal therapeutic and/or formulation characteristics prior to or concurrently with the generation of the full protein improves the overall drug development efficiency. This approach thus allows generation of proteins of both high therapeutic efficacy and optimal formulation characteristics, *e.g.*, high solubility and long shelf life.

[0013] The invention provides an integrated approach to therapeutic protein development which incorporates therapeutic and/or formulation and/or manufacturing considerations in the early screening and development process. The approach involves evaluating one or more individual domains of a protein to identify domains that have both the desired biological activity and desired therapeutic and/or formulation characteristics. For example, a plurality of different variants of a domain that have been determined to have the desired biological activity can be screened to identify one or more variants that have certain

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desired therapeutic and/or formulation characteristics, *e.g.*, having desired stability and/or solubility. The identified domain variants are then used in the construction of the full multidomain proteins. For example, in therapeutic antibody development, after affinity screening of an expression library (*e.g.*, a phage display library), Fab domains having high binding affinity can be screened for their formulation characteristics, *e.g.*, solubility and/or stability. One or more Fab domains having desired formulation characteristics, *e.g.*, having high solubility and/or stability, are selected and each used with an appropriate Fc domain in the construction of the full antibodies. Different candidate (including variant/modified domains) Fc domains can also be screened for formulation characteristics, *e.g.*, solubility and stability, to select an Fc domain that has the desired formulation characteristics. The constructed antibodies are then submitted for further formulation development.

[0014] The invention further provides methods for the development of a therapeutic multidomain protein by engineering one or more domains of the multidomain protein to have the desired therapeutic and/or formulation characteristics such as for example, biodistribution, non-specific toxicity, solubility and/or stability. For example, a domain having a desired biological activity can be modified by replacing one or more amino acid residues in said domain to generated a modified domain or a population of modified domains which are then screened for their therapeutic characteristics, *e.g.*, biodistribution and/or non-specific toxicity. One or more modified domains having desired therapeutic characteristics are selected and each used in the construction of the full multidomain protein.

[0015] The present invention also provides a method for evaluating the shelf life, i.e., the long term stability, of a protein preparation comprising a multidomain protein based on a thermal denaturation and/or renaturation behavior of a multidomain protein in a liquid protein preparation. It is contemplated that the multidomain protein may comprise a domain whose unfolding leads to aggregation of the multidomain protein in the liquid protein preparation. The liquid protein preparation can be a solution of the multidomain protein of a particular concentration, *e.g.*, a concentration from about 5 to 300 mg/ml. The liquid protein preparation can also comprise other substances, including but not limited to, salts, ligands, co-factors, and so on. The method thus can also be used for determining the optimal conditions, *e.g.*, the constituents and the optimal concentration of each such constituent.

[0016] The invention provides a method of generating one or more multidomain proteins, comprising (a) evaluating for each of a plurality of candidate domains one or more metrics representing one or more therapeutic and/or formulation and/or manufacturing

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characteristics of the domain, wherein the plurality of candidate domains exhibits a biological activity above a predetermined threshold level; (b) selecting one or more domains from the plurality based on the metrics; and (c) optionally, constructing a full multidomain protein using each domain selected in step (b) and one or more other domains. In one embodiment, the method of the invention is used to generate one or more antibodies. In a specific embodiment, the domain is an antigen binding domain (*e.g.*, Fab domain). In one embodiment, the metrics of the invention include one or more parameters characterizing the stability of a candidate domain. In another embodiment, the metrics of the invention include one or more parameters characterizing the solubility, biodistribution or non-specific toxicity of a candidate domain. In a specific embodiment, the parameters characterizing stability of a candidate domain include the Tm value of the domain, and the parameters characterizing solubility, biodistribution or non-specific toxicity of a candidate domain include the pI value of the domain. The metrics of the invention may further include metrics characterizing stability of a candidate domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles.

[0017] The plurality of candidate domains can be an antigen binding domains (*e.g.*, Fab domains). In such cases, the biological activity may be the antigen binding domain's binding affinity to a target antigen. In certain embodiments, at least some of the antigen binding domains bind different epitopes of the antigen. In a specific embodiment, the plurality of antigen binding domains are obtained by screening an expression library (*e.g.*, a phage display library) with the antigen. In another specific embodiment, the plurality of antigen binding domains are obtained by digesting a plurality of monoclonal antibodies. Each selected antigen binding is then combined with one or more other domains (*e.g.*, constant region domains) to generate one or more multidomain proteins.

[0018] The plurality of candidate domains can be constant region domains. In such cases, the biological activity may be the constant region domain's binding affinity to constant region domain receptors and/or ligands (*e.g.*, FcRn, C1q, FcγRs) and/or ability to mediate effector functions (*e.g.*, ADCC, CDC). In a specific embodiment, the plurality of constant region domains are obtained by screening an expression library with a constant region domain ligand. Each selected constant region domain is then combined with another domain (*e.g.*, a antigen binding domain, a cellular receptor domain) to generate one or more multidomain proteins.

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[0019] In one embodiment, metrics of the invention include one or more parameters characterizing stability of an antigen binding domain (*e.g.*, Fab domain). In another embodiment, metrics include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of an antigen binding domain. In a specific embodiment, the parameters characterizing stability of an antigen binding domain include a Tm value of the antigen binding domain, and the parameters characterizing solubility, biodistribution or non-specific toxicity of an antigen binding domain comprise a pI value of the antigen binding domain. The metrics of the invention may further include metrics characterizing stability of the antigen binding domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles.

[0020] In one embodiment, metrics of the invention include one or more parameters characterizing stability of a constant region domain (*e.g.*, Fc domain). In another embodiment, metrics include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of a constant region domain. In a specific embodiment, said the parameters characterizing stability of said constant region domain include a Tm value of the constant region domain, and the parameters characterizing solubility, biodistribution or non-specific toxicity of the constant region domain include a pI value of the constant region domain. The metrics of the invention may further include metrics characterizing stability of the constant region domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles.

[0021] In one embodiment, the method of generating one or more multidomain proteins further comprises, before using the selected domain to construct a full multidomain protein (prior to said step (c)), the steps of (i) evaluating for each of a plurality of other candidate domains one or more metrics; and (ii) selecting another domain from the plurality of other candidate domains based on the metrics. In one embodiment, the metrics comprise one or more parameters characterizing stability of the second domain. In another embodiment, the metrics of the invention include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of the second domain. In one embodiment, the parameters characterizing stability of the second domain include a Tm value of the second domain, and the parameters characterizing solubility, biodistribution or non-specific toxicity of said second domain include a pI value of the second domain. The metrics of the invention

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may further include metrics characterizing stability of the second domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles. In one embodiment, the second domain is a constant region domain. In a specific embodiment, the first domain is an antigen binding domain (*e.g.*, Fab domain) and the second domain is an antibody constant region domain (*e.g.*, Fc domain).

[0022] In a specific embodiment, the invention provides a method of generating one or more antibodies which bind to a given target, comprising (a) identifying a plurality of candidate antigen binding domains exhibiting a binding affinity for the target above a predetermined threshold level; (b) determining the Tm and/or pI values for each of the plurality of candidate antigen binding domains; (c) selecting one or more antigen binding domains from the plurality based on their Tm and/or pI values; and (d) constructing an antibody using each antigen binding domain selected in step (c) and one or more other domains. In one embodiment, at least some of the antigen binding domains bind different epitopes of the target.

[0023] The invention also provides a method of screening one or more multidomain proteins, such as antibodies, which bind to a given target, comprising (a) evaluating for each of a plurality of multidomain proteins one or more metrics of the invention representing one or more therapeutic and/or formulation and/or manufacturing characteristics of candidate domains of the multidomain proteins, wherein the plurality of multidomain proteins exhibits a biological activity above a predetermined threshold level; and (b) selecting one or more multidomain proteins from the plurality based on the metrics representing one or more therapeutic and/or formulation and/or manufacturing characteristics of said multidomain proteins.

[0024] The invention also provides a method of engineering a multidomain protein to improve one or more therapeutic and/or formulation and/or manufacturing characteristics without significantly altering other biological activities. The method comprises making one or more modifications (*e.g.*, amino acid substitutions), wherein the modifications (*e.g.*, amino acid substitutions) are such that they improve one or more of the characteristics of the multidomain protein.

[0025] In the method of engineering a multidomain protein to have improved improve one or more therapeutic and/or formulation and/or manufacturing characteristics without significantly altering other biological activities, the multidomain protein can be an antibody

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and the biological activities include, but are not limited to, one or more of the following biological activities: antigen binding, serum half-life, complement fixation, Fc receptor binding and antigen-dependent cytotoxicity of the antibody.

[0026] The invention also provides a method of evaluating long term stability of a multidomain protein in a solution, wherein long term stability is defined as less than 5%, 10%, or 20% aggregation upon storage for a predetermined period of time (*e.g.*, 1 to 6 months) at a predetermined temperature (*e.g.*, 4°C). The method comprises (a) providing a solution of a multidomain protein; (b) denaturing one or more domains of a multidomain protein by heating the solution of the multidomain protein; (c) determining if the domains refold upon cooling; and (d) classifying the multidomain protein as having long term stability in the solution if the domains are determined to refold in step (c).

[0027] The invention also provides a method of identifying in a multidomain protein one or more domains, the unfolding of which causes aggregation of the multidomain protein in a solution, comprising (a) denaturing the one or more domains by heating the solution of the multidomain protein; (b) determining if one or more of the domains refold upon cooling; and (c) identifying one or more domains that do not refold upon cooling in step (b), thereby identifying a domain or domains, the unfolding of which causes aggregation of the multidomain protein in the solution.

[0028] The invention also provides a method of engineering a multidomain protein to have improved long term stability in a solution, wherein said long term stability is defined as less than 5%, less than 10%, or less than 20% aggregation upon storage for a predetermined period of time at a predetermined temperature. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) denaturing the modified domain by heating a solution of a multidomain protein comprising the modified domain; (c) determining if the modified domain in the multidomain protein refolds upon cooling; and (d) classifying the multidomain protein containing the modified domain as having improved long term stability if the modified domain is determined to refold in step (c). In certain embodiments, the unfolding of the domain prior to modification causes the aggregation of the multidomain protein. In the method of engineering a multidomain protein to have improved long term stability, the multidomain protein can be an antibody. It is contemplated that steps (a) to (d) may be performed on an isolated domain. For example, a Fab domain may be isolated, modified denatured, renatured and classified. In one embodiment, the identified domain having

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improved long term stability is used in the generation of a multidomain protein. In another embodiment, the modified domain is an antigen binding domain (*e.g.*, Fab domain). In still another embodiment, the modified domain is a constant region domain (*e.g.*, Fc domain).

[0029] The invention further provides a method of screening for a multidomain protein which has improved long term stability in a solution, wherein long term stability is defined as less than 5%, 10%, or 20% aggregation upon storage for a predetermined period of time at a predetermined temperature comprising (a) denaturing respectively two or more multidomain proteins that are members of a population of multidomain proteins, wherein each multidomain protein comprises a different modified domain, the modified domains having one or more amino acid residues substituted, wherein the denaturing is accomplished by heating a solution of one of the multidomain proteins comprising a modified domain; (b) determining if the modified domain in each of the multidomain proteins denatured in step (a) refolds upon cooling; and (c) identifying the multidomain proteins containing a modified domain determined to refold in step (b) as having improved long term stability. In one embodiment, the method further comprises generating the population of multidomain proteins comprising different modified domains. In certain embodiments the unfolding of the domain prior to modification causes aggregation of the multidomain protein.

[0030] In the method of screening for a multidomain protein which has improved long term stability, the multidomain protein can be an antibody. In one embodiment, the domain is an antigen binding domain (*e.g.*, a Fab domain). In another embodiment, the domain is a constant region domain (*e.g.*, a Fc domain). It is contemplated that the methods of the invention may be performed on an isolated domain or domains. In one embodiment, the modified domain determined to refold upon cooling is used in the generation of a multidomain protein. In one embodiment, said denaturation and renaturation steps are carried out by generating a thermal denaturation/renaturation curve, *e.g.*, using differential scanning calorimetry.

[0031] The invention provides a method of engineering a multidomain protein to have improved stability. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the Tm of the modified domain; and (c) classifying the multidomain protein containing the modified domain as having improved stability if the domain is determined to have a higher Tm in step (b). Accordingly, the invention also provides engineered multidomain proteins which have a higher Tm than the multidomain protein prior to being

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engineered. The invention also provides engineered multidomain proteins which have improved stability than the multidomain protein prior to being engineered. In certain embodiments, the Tm of the domain prior to modification is either low or contributes to the multidomain protein have a low Tm.

[0032] The invention provides a method of engineering a multidomain protein to have improved solubility and/or lower viscosity. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying the multidomain protein containing the modified domain as having improved solubility and/or viscosity if the modified domain is determined to have a higher pI in step (b). In another embodiment of the method, the pI of the multidomain protein containing the modified domain is determined in step (b) and the multidomain protein containing the modified domain is classified as having improved solubility and/or viscosity if the multidomain protein containing the modified domain is determined to have a higher pI in step (b). Accordingly, the invention also provides engineered multidomain proteins which have a higher pI relative to the multidomain protein prior to being engineered. The invention also provides engineered multidomain proteins which have improved solubility and/or lower viscosity relative to the multidomain protein prior to being engineered. In certain embodiments, the pI of the domain prior to modification is either low or contributes to the multidomain protein have a low pI.

[0033] The invention further provides a method of engineering a multidomain protein to have reduced non-specific toxicity. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying the multidomain protein containing the modified domain as having reduced non-specific toxicity if the modified domain is determined to have a lower pI in step (b). In another embodiment of the method, the pI of the multidomain protein containing the modified domain is determined in step (b) and the multidomain protein containing the modified domain is classified as having reduced non-specific toxicity if the multidomain protein containing the modified domain is determined to have a lower pI in step (b). Accordingly, the invention also provides engineered multidomain proteins which have a lower pI relative to the multidomain protein prior to being engineered. The invention also provides engineered multidomain proteins which have reduced non-specific toxicity relative to the multidomain

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protein prior to being engineered. In certain embodiments, the domain prior to modification either has a pI or contributes to the multidomain protein having a pI which results in non-specific toxicity.

[0034] In addition, the invention provides a method of engineering a multidomain protein to have a specific biodistribution (*e.g.*, intracellular, extravascular, extracellular). The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying the multidomain protein containing the modified domain as having reduced intracellular localization if the modified domain is determined to have a lower pI in step (b) or as having increased intracellular and/or extravascular localization if the modified domain is determined to have a higher pI in step (b). In another embodiment of the method, the pI of the multidomain protein containing the modified domain is determined in step (b) and the multidomain protein containing the modified domain is classified as having reduced intracellular localization if the multidomain protein containing the modified domain is determined to have a lower pI in step (b) or as having increased intracellular and/or extravascular localization if the multidomain protein containing the modified domain is determined to have a higher pI in step (b). Accordingly, the invention also provides engineered multidomain proteins which have a specific biodistribution. Specifically, the invention provides engineered multidomain proteins which have increased intracellular and/or extravascular localization relative to the multidomain protein prior to being engineered. The invention also provides engineered multidomain proteins which have reduced intracellular localization relative to the multidomain protein prior to being engineered. In certain embodiments, the domain prior to modification either has a pI or contributes to the multidomain protein having a pI which determines the biodistribution.

4. BRIEF DESCRIPTION OF THE FIGURES

[0035] FIG. 1. The DSC thermogram of the full length Ca-hG1 Mab molecule is shown in the top panel, while the bottom panel is an overlay of the thermograms obtained from purified Fab and Fc fragments of Ca-hG1 Mab molecule. Two discrete peaks are seen for the Fc domain at approximately 68°C and 83°C. A single peak is seen for the Fab fragment at approximately 87°C.

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[0036] FIG. 2. Deconvolution of the DSC thermogram of the full length Ca-hG1 Mab (see Fig. 1). Three discrete peaks are seen with Tm values of 60°C, 83°C and 87°C.

[0037] FIG. 3. Overlay of multiple DSC scans of the full length Ca-hG1 Mab, demonstrating reversibility over multiple heating and cooling cycles when heated to 72°C.

[0038] FIG. 4. Overlay of multiple DSC scans of the full length Ca-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 87°C than seen for heating to 72°C (see FIG. 3).

[0039] FIG. 5. Overlay of multiple DSC scans of the Fab fragment of Ca-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 85°C than seen for heating to 72°C (see FIG. 3).

[0040] FIG. 6. Overlay of multiple DSC scans of the Fab fragment of Ca-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 87°C than seen for heating to 68°C (see FIG. 7).

[0041] FIG. 7. Overlay of multiple DSC scans of the Fc fragment of Ca-hG1 Mab, demonstrating reversibility over multiple heating and cooling cycles when heated to 68°C.

[0042] FIG. 8. Overlay of multiple DSC scans of the Fc fragment of Ca-hG1 Mab, demonstrating poor reversibility over multiple heating and cooling cycles when heated to 83°C than seen for heating to 68°C (see FIG. 7).

[0043] FIG. 9. Overlay of DSC scans of full length Mabs Ba-hG1 and Ca-hG1 showing dramatically different profiles. Ba-hG1 has a major peak at ~72°C while the major peak for Ca-hG1 is seen at ~87°C.

[0044] FIG. 10. The DSC thermogram of the full length Ba-hG1 Mab molecule is shown in the top panel, while the bottom panel is an overlay of the thermograms obtained from purified Fab and Fc fragments of Ba-hG1 Mab molecule. Two discrete peaks are seen for the Fc domain at approximately 67°C and 83°C. Two overlapping peaks are seen for the Fab fragment at approximately 72°C and 76°C.

[0045] FIG. 11. Deconvolution of the DSC thermogram of the full length Ba-hG1 Mab (see Fig. 10). Three discrete peaks are seen with Tm values of 71°C, 82°C and 93°C.

[0046] FIG. 12. Overlay of multiple DSC scans of full length Ba-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 70°C than seen for the Fab fragment alone heated to 71°C (see FIG. 14).

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[0047] FIG. 13. Overlay of multiple DSC scans of full length Ba-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 73°C than seen for the Fab fragment alone heated to 71°C (see FIG. 14).

[0048] FIG. 14. Overlay of multiple DSC scans of the Fab fragment of Ba-hG1 Mab, demonstrating reversibility over multiple heating and cooling cycles when heated to 71°C.

[0049] FIG. 15. Overlay of multiple DSC scans of the Fab fragment of Ba-hG1 Mab, demonstrating less reversibility over multiple heating and cooling cycles when heated to 76°C than seen for heating to 71°C (see FIG. 14).

[0050] FIG. 16. Overlay of multiple DSC scans of the Fc fragment of Ba-hG1 Mab, demonstrating reversibility over multiple heating and cooling cycles when heated to 67°C.

[0051] FIG. 17. Graphical representation of the IEF and DSC analysis for a different panel of antibodies. Antibodies Ca-hG1, Ca1-hG1 and Ca1-hG1a are highly related, recognizing the same protein epitope and differing by only a few amino acids. The remaining antibodies, As-hG1, Ba-hG1 and Da-hG1 are unrelated.

[0052] FIG. 18. Analysis of a panel of different antibodies which bind to a variety of epitopes present on a single protein. These antibodies have a common Fc domain and vary only in their Fab regions A) Isoelectric focusing (IEF) demonstrate that there is a wide range of pI values (*e.g.*, ~7.8 to ~9.2) for the different antibodies. B) Differential scanning calorimetry (DSC) analysis indicates that there is a wide range of Tm values (*e.g.*, ~66°C to ~90°C) for the different antibodies. C) Graphical representation of the IEF and DSC analysis for the panel of antibodies.

[0053] FIG. 19. Graphical representation of the IEF and DSC analysis for a different panel of antibodies which bind to a variety of epitopes present on a single protein. Also plotted are several antibodies used in earlier studies and two chimeric antibodies generated by combining the heavy and light chains from two different antibodies. All of these antibodies have a common Fc domain and vary only in their Fab regions.

[0054] FIG. 20. Graphical representation of the IEF and DSC analysis of a panel of antibodies which are identical except for mutations in their hinge region.

[0055] FIG. 21. Viscosity analysis for several of the antibodies analyzed in Fig 17. Each antibody was analyzed at 100 mg/ml, in addition Ba-hG1 was analyzed at 126 mg/ml, 138 mg/ml and 150 mg/ml.

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[0056] FIG. 22. Aggregation rate vs. Fab Tm. Panel A plots the aggregation rate of several Ba-hG1, Da-hG1, Ca-hG1 and Ca1-hG1 in relation to the Tm of the Fab region. Panel B plots the aggregation over time for the isolated Fc region and Fab domains of Ca-hG1 and Ba-hG1.

[0057] FIG. 23. Antibody naming scheme used in this application. First 3 characters identify the antigen binding domain: the capital letter designates the target, the lower case letter designates a particular epitope of the target, the number designates the variant of the antibody (for simplicity reasons, 0 is often omitted). The last 4 characters identify the Fc domain: the first 3 characters identify the Fc domain, the last lower case letter designates the Fc variant. The example in FIG. 23, Aa1-hG1a, thus identifies variant "1" of an antibody that binds epitope "a" of target "A" and has variant "a" of a human gamma-1 (hG1) as its Fc domain.

[0058] FIG. 24. Ribbon diagram of CH2 and CH3 of the human Fc showing the location of the initial five residues where substitutions were made. This illustration is a side view of a human Fc showing only one CH2 domain (top) and one CH3 domain (bottom). Residues that were substituted are spacefilled. The red indicates a region where mutations may affect C1q, FcγR, FcRn, and protein A binding, the blue indicates a region where mutations may affect FcγR and FcRn binding, and the green indicates a region where mutations may affect FcRn and protein A binding. The yellow region is where the two CH3 domains pack against each other.

[0059] FIG. 25. Ribbon diagram of CH2 and CH3 of the human Fc showing the location of six additional residues where substitutions were made. The orientation is the same as in Figure 1 and the coloration conveys the same meaning as described in Figure 1. Substituted residues are spacefilled.

[0060] FIG. 26. IEF gel of the first ten single substitutions made. The pI was determined by measuring the migration of the band with the highest intensity in a given lane. The wild type antibody control (WT) was produced in NSO cells.

[0061] FIG. 27. IEF gel of the double and triple substitutions. The triple substitutions E345K Q362K D401N and E345K Q362K D401K probably have too high of a pI to be resolved on this gel and ran off. WT antibody produced both in 293H and NSO cells were run for comparison.

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[0062] FIG. 28. IEF gel of the second 12 single substitutions made. The pI was determined by measuring the migration of the band with the highest intensity in a given lane. wild type (WT) antibody produced both in 293H and NSO cells were run for comparison.

[0063] FIG 29. Correlation of pI shift vs. nominal charge change. Plotted are the values for the delta change in pI vs. the delta change in the charge for each of the substituted antibodies. The correlation is largely linear.

[0064] FIG 30. Overlay of DSC thermograms of the wild type and single substituted anti-IL9 antibodies with altered pI. The Tm profiles of E345Q, E345K, R355Q, R355E, Q362E and Q362K(in the left panel) are very similar to that of the wild type antibody each having one major peak at ~76°C. The Tm profiles of K340Q, K340E and D401K have a distinct peak between about 62°C and ~70°C in addition to the major peak at ~76°C.

[0065] FIG 31. Overlay of DSC thermograms of the wild type and triple substituted anti-IL9 antibodies with altered pI.

[0066] FIG 32. Overlay of DSC thermograms of the wild type Fa-hG1 antibody and the two variants Fa-hG1b and Fa-hG4a. The major peak representing the Tm of the Fab domain is at 70.4°C for both Fa-hG1 and Fa-hG4a and at 70.9°C for Fa-hG1b.

[0067] FIG 33. IEF gel of the wild type Fa-hG1 antibody and the two variants Fa-hG1b and Fa-hG4a. The pI of the major protein bands are 8.35, 8.18 and 713 for Fa-hG1, Fa-hG1b and Fa-hG4a, respectively.

5. DETAILED DESCRIPTION OF THE INVENTION

[0068] The invention is based in part on the discovery of the inventors that certain therapeutic and/or formulation and/or manufacturing characteristics of a multidomain protein (*e.g.*, antibody) can be evaluated by examining individual domains that form the protein. One or more metrics representing the physical and/or chemical and/or structural characteristics of protein domains are determined and individual domains are then selected based on their metrics. The individual domains may be examined in the context of the intact multidomain protein or as isolated domains. For example, the physical/chemical (including structural) characteristics of a Fab or an Fc domain in an antibody may determine the biodistribution and/or non-specific toxicity and/or stability and/or solubility and/or viscosity of the antibody. An approach incorporating selecting appropriate domains having optimal therapeutic and/or formulation characteristics prior to or concurrently with the generation of the full protein improves the overall drug development efficiency. This approach thus allows generation of

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proteins of both high therapeutic efficacy and optimal formulation characteristics, *e.g.*, high solubility and long shelf life.

[0069] The invention provides an integrated approach to therapeutic protein development which incorporates therapeutic and/or formulation and/or manufacturing considerations in the early screening and development process. The approach involves evaluating one or more individual candidate domains of a protein to identify domains that have both the desired biological activity and desired therapeutic and/or formulation characteristics. For example, a plurality of different variants of a domain (also encompassed by the terms "domain variant(s)," "variant domain(s)" and "candidate domain(s)") that have been determined to have the desired biological activity can be screened to identify one or more variants that have certain desired therapeutic and/or formulation characteristics based on certain metrics (*e.g.*, Tm and/or pI). The identified domain variants are then used in the construction of the full multidomain proteins. For example, in therapeutic antibody development, after affinity screening of an expression library (*e.g.*, a phage display library), candidate Fab domains having high binding affinity can be screened for their formulation characteristics, *e.g.*, solubility and/or stability. One or more Fab domains having desired formulation characteristics, *e.g.*, having high solubility and/or stability, are selected and each used with an appropriate Fc domain in the construction of the full antibodies. Different candidate Fc domains can also be screened for formulation characteristics, *e.g.*, solubility and stability, to select an Fc domain that has the desired formulation characteristics. The constructed antibodies are then submitted for further formulation development.

[0070] The invention further provides methods for the development of a therapeutic multidomain protein by engineering one or more domains of the multidomain protein to have the desired therapeutic and/or formulation characteristics such as for example, biodistribution, non-specific toxicity, solubility and/or stability. For example, a domain having a desired biological activity can be modified by replacing one or more amino acid residues in said domain to generated a modified domain (also encompassed by the terms "domain variant(s)," "variant domain(s)" and "candidate domain(s)") or a population of modified domains which are then screened for their therapeutic characteristics, *e.g.*, biodistribution and/or non-specific toxicity by comparing certain metrics (*e.g.*, Tm and/or pI) of the modified domains to those of the unmodified domain. One or more modified domains having desired therapeutic characteristics based on their metrics (*e.g.*, having a desired Tm and/or pI) are selected and each used in the construction of the full multidomain protein.

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[0071] The present invention also provides a method for evaluating the shelf life, i.e., the long term stability, of a protein preparation comprising a multidomain protein based on a thermal denaturation and/or renaturation behavior of a multidomain protein in a liquid protein preparation. It is contemplated that the multidomain protein may comprise a domain whose unfolding leads to aggregation of the multidomain protein in the liquid protein preparation. The liquid protein preparation can be a solution of the multidomain protein of a particular concentration, *e.g.*, a concentration from about 5 to 300 mg/ml. The liquid protein preparation can also comprise other substances, including but not limited to, salts, ligands, co-factors, and so on. The method thus can also be used for determining the optimal conditions, *e.g.*, the constituents and the optimal concentration of each such constituent.

[0072] The inventor has discovered that the aggregation of a multidomain protein in a solution is the result of thermal unfolding (also referred to herein as "thermal denaturation" or simply "denaturation") of a particular domain or domains in the protein, and that thermal denaturation and/or renaturation behavior of a multidomain protein comprising the particular domain or domains provides an indication of the long term stability and, thus, the shelf life, of a liquid formulation of the multidomain protein.

[0073] The pI of certain therapeutic multidomain proteins is known to affect their biodistribution and/or their non-specific toxicity profiles. The inventor has further discovered that the viscosity and solubility of a multidomain protein in a solution correlates with the pI of the multidomain protein and/or a particular domain or domains in the protein, relative to the pH of the solution. Thus, the pI of a multidomain protein and/or one or more individual domains provides an indication of viscosity and solubility of a liquid formulation of the multidomain protein in addition to other therapeutic characteristics. Additionally, the inventor has discovered that the pI of multidomain proteins can be modified to alter those characteristics which are governed by the pI of a protein.

5.1. Methods For Development Of Multidomain Protein Therapeutics

[0074] The invention provides a method for protein drug development that incorporates therapeutic and/or formulation and/or manufacturing considerations in the early screening process. A protein drug is a formulated drug form of a therapeutic protein which can be used in the treatment or prevention of a disease. The formulation of the drug aids and/or optimizes the clinical effect of the protein, i.e., the pharmacological effect of the protein. The development of a therapeutic protein depends on the physical, chemical, and

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pharmacokinetic properties of the protein. The manufacturing process of a protein drug, including production, purification, storage, and any downstream processing of the protein, also depends on the physical and chemical properties of the protein.

[0075] As used herein, the "therapeutic characteristics" of a protein include physical, chemical, and pharmacokinetic properties of the protein which affect the pharmacological effect of the protein drug form. Examples of therapeutic characteristics of a protein include, but are not limited to, biodistribution, non-specific toxicity and clearance profiles of the protein.

[0076] As used herein, the formulation and/or manufacturing characteristics of a protein include physical, chemical, and pharmacokinetic properties of the protein which affect the pharmacological effect of the protein drug form and/or the production, purification, storage and any downstream processing of the protein. For simplicity reasons, the formulation and/or manufacturing characteristics of a protein are often referred to herein jointly as simply the "formulation characteristics." Examples of formulation characteristics of a protein include, but are not limited to, stability (*e.g.*, thermal stability and/or storage stability, also termed shelf life), solubility and viscosity of the protein.

[0077] The invention provides a method for the generation of multidomain proteins having both high biological activities and optimal therapeutic and/or formulation and/or manufacturing characteristics. The method comprises evaluating the biological activities and the therapeutic and/or formulation and/or manufacturing characteristics of individual domains of a multidomain protein, and identifying domains that exhibit both desired biological activities, *e.g.*, desired level of target binding affinity and/or desired serum half-life, etc., and desired therapeutic and/or formulation and/or manufacturing characteristics, *e.g.*, desired level of solubility and/or stability. In certain embodiments, the therapeutic and/or formulation and/or manufacturing characteristics of individual domains of a multidomain protein are evaluated by metrics of the invention which include one or more parameters characterizing therapeutic and/or formulation characteristics including but not limited to stability, solubility, biodistribution and non-specific toxicity. In certain embodiments, individual domains that exhibits an optimal combination of biological activities and therapeutic and/or formulation and/or manufacturing characteristics are identified. In certain other embodiments, different domains which when combined in a protein exhibit an optimal combination of biological activities and therapeutic and/or formulation and/or manufacturing

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characteristics are identified. The identified domains are then used to construct the full protein.

[0078] In one embodiment, the method of generating one or more multidomain proteins for therapeutic uses, comprises (a) evaluating for each of a plurality of candidate domains one or more metrics representing one or more therapeutic and/or formulation and/or manufacturing characteristics of the first domain, wherein the plurality of candidate domains exhibits a biological activity above a predetermined threshold level; (b) selecting one or more domains from the plurality based on the metrics; and (c) optionally, constructing a full multidomain protein using each domain selected in step (b) and one or more other domains. In one embodiment, the metrics include one or more parameters characterizing stability of the candidate domain. In another embodiment, the metrics include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of the candidate domain.

[0079] Thus, the present invention provides a method comprising screening a plurality of candidate domains to identify one or more domains that have both the desired biological activities, *e.g.*, desired target binding affinity, and desired therapeutic and/or formulation characteristics, *e.g.*, biodistribution, non-specific toxicity, stability, solubility and viscosity. Each of the candidate domains is also referred to as a variant of the domain. The method comprises evaluating the candidate domains for both their biological activities and their therapeutic and/or formulation properties. One or more candidate domains which exhibit an optimal combination of biological activities and therapeutic and/or formulation characteristics are selected for constructing the full multidomain proteins.

[0080] In one embodiment, the method of generating one or more multidomain proteins for therapeutic uses further comprises, before using the selected domain to construct a full multidomain protein (prior to said step (c)), the steps of (i) evaluating for each of a plurality of other candidate domains one or more metrics; and (ii) selecting a another domain from the plurality of other candidate domains based on the metrics. In one embodiment, the metrics include one or more parameters characterizing stability of the other candidate domain. In another embodiment, the metrics include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of the other candidate domain.

[0081] The present invention also provides a method for screening a plurality of candidate domains to identify one or more domains that have both the desired biological activities, *e.g.*, desired target binding affinity, and desired metrics representing their therapeutic and/or formulation characteristics. in the context of the full multidomain protein.

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Thus, the present invention provides a method to evaluate one or more domains of a multidomain protein in an intact multidomain protein. By screening a plurality of intact multidomain proteins for one or more candidate domains which exhibit an optimal combination of biological activities therapeutic and/or formulation characteristics, the need to construct the full multidomain protein is eliminated. In one embodiment, the method comprises screening a plurality of antigen binding domains that bind a given target antigen in the context of an intact full length antibody molecule, to identify one or more antigen binding domains that have desired therapeutic and/or formulation characteristics.

[0082] The present invention further provides a method to engineer a domain to improve one or more therapeutic and/or formulation characteristic. In one embodiment, specific modifications (*e.g.,* amino acids substitutions, additions or deletions) are engineered into a domain to modify one or more desired therapeutic and/or formulation characteristics.

[0083] In one embodiment, the formulation characteristics of a candidate protein domain is evaluated by evaluating one or more metrics of the invention (also referred to herein as simply "metrics") characterizing the formulation characteristics of the candidate domain. In one embodiment, the metrics include one or more parameters characterizing the stability of a domain. In another embodiment, the metrics include one or more parameters characterizing the solubility, biodistribution or non-specific toxicity of a domain. In a specific embodiment, the one or more parameters characterizing stability of the domain include a Thermal melting temperature (Tm) value of the domain. The Tm of a candidate domain, *e.g.*, a Fab domain, can be a good indicator of the thermal stability of a protein containing the domain and may further provide an indication of the shelf-life of a protein containing the domain. A lower Tm indicates more aggregation/less stability, whereas a higher Tm indicates less aggregation/ more stability. Thus, candidate domains having higher Tm are preferable for incorporation into a full multidomain protein (*e.g.*, an antibody). In one embodiment, candidate domains having a Tm greater than a predetermined threshold value are selected. In some embodiments, one or more candidate domains that have a Tm value higher than at least 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C, 105°C, 110°C, 115°C or 120°C are selected for construction of the full multidomain protein. In specific embodiments, the candidate domains are antigen binding domains (*e.g.*, Fab domain, scFv, etc.).

[0084] Thermal melting temperatures (Tm) of a protein domain can be measured using any standard method known in the art. For example, Vermeer *et al.* studied the

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unfolding and denaturation of a monoclonal mouse anti-rat IgG of isotype 2b by differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy (Vermeer *et al.*, 2000, Biophys. J. 78:394-404; Vermeer *et al.*, 2000, Colloids Surfaces A: Physicochem. Eng. Aspects. 161:139-150; Vermeer *et al.*, 2000, J. Colloid Interface Sci. 225:394-397; Vermeer *et al.*, 2000, Biophys. J. 79:2150-2154). They showed that the folding/unfolding of the IgG can be characterized by two main transitions that are themselves superpositions of various steps. The bimodal distribution observed in both DSC and CD experiments did not depend on the scan rate in the experiments. The two transitions appeared to be independent, and the unfolding was irreversible. The IgG was then digested into isolated Fab and Fc fragments (Vermeer *et al.*, 2000, Biophys. J. 79: 2150-2154). The secondary structure as well as the thermodynamic stability of the two isolated fragments were studied and compared with those of the intact immunoglobulin. It was shown that the two peaks observed for intact IgG can be assigned to the Fab and Fc fragments, respectively. Vermeer *et al.* also showed that, in addition to induction by heat, the structural perturbation of IgG in general could also be triggered by changing the pH (Vermeer *et al.*, 2000, Biophys. J. 78:394-404) or by interaction with a hydrophobic environment, *e.g.*, adsorption onto Teflon surfaces or interaction with surfactants (Vermeer *et al.*, 1998, Biochim. Biophys. Acta. 1425:1-12; Vermeer *et al.*, 2000, Colloids Surfaces A: Physicochem. Eng. Aspects. 161:139-150; Vermeer *et al.*, 2000, J. Colloid Interface Sci. 225:394-397).

[0085] In one embodiment, the Tm of a protein domain is measured using a sample containing isolated protein domain molecules. In another embodiment, the Tm of a protein domain is measured using a sample containing an intact multidomain protein that contains the domain to be analyzed. In the latter case, the Tm of the domain is deduced from the data of the protein by analyzing only those data points corresponding to the domain of interest. In one embodiment, the Tm of a protein or protein domain is measured with a VP-DSC (MicroCal, LLC) using a scan rate of 1.0°C/min and a temperature range of 25 –120°C. A filter period of 8 seconds is used along with a 5 minute pre-scan thermostating. In a specific example, samples are prepared by dialysis into 25 mM Histidine-HCl, pH 6 using Pierce dialysis cups (3.5 kD). Average Mab concentrations are 50 μg/mL as determined by A280. Melting temperatures are determined following manufacturer procedures using Origin software supplied with the system. Briefly, multiple baselines are run with buffer in both the sample and reference cell to establish thermal equilibrium. After the baseline is subtracted from the sample thermogram, the data are concentration normalized and fitted using the

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deconvolution function. In another embodiment, stability of the candidate domains are evaluated using a method described in Section 5.2. The one or more metrics may further include metrics characterizing stability of the domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles.

[0086] In another specific embodiment, the one or more parameters characterizing the solubility, biodistribution or non-specific toxicity of the domain includes an Isoelectric Point (pI) value of the domain. The pI of a protein is defined as the pH at which a polypeptide carries no net charge. It is known in the art that protein solubility is typically lowest when the pH of the solution is equal to the isoelectric point (pI) of the protein. It is thus possible to evaluate the solubility of a protein for a given pH, *e.g.*, pH 6, based on its pI. The pI of a protein is also a good indicator of the viscosity of the protein in a liquid formulation. High pI indicates high solubility and low viscosity (especially important for high concentration formulations). In one embodiment, candidate domains having a pI greater than a predetermined threshold value are selected. As described above, the pI of a protein also plays a role in biodistribution and non-specific toxicity of multidomain proteins. For example, it is known in the art that reducing the pI of recombinant toxins results in lower non-specific toxicity and renal accumulation. Alternatively, increases the pI of antibodies is known to increase their intracellular and/or extravascular localization. One of skill in the art can readily determine which therapeutic characteristics are most desirable for a particular multidomain protein. In some embodiments, one or more candidate domains that have a pI value higher than about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0 are selected for construction of the full multidomain protein. In other embodiments, one or more candidate domains that have a pI value less than about 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, or 5.0 are selected for construction of the full multidomain protein. It will be understood by one skilled in the art that a single protein will have multiple charge forms. Without wishing to be bound by any particular theory, the charge of a protein can be modified by a number of different mechanisms including but not limited to, amino acid substitution, cationization, deamination, carboxyl-terminal amino acid heterogeneity, phosphorylation and glycosylation. As used herein the pI value is defined as the pI of the predominant charge form.

[0087] The pI of a protein may be determined by a variety of methods including but not limited to, isoelectric focusing and various computer algorithms (see for example Bjellqvist *et al.*, 1993, *Electrophoresis* 14:1023). In one embodiment, pI is determined using

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a Pharmacia Biotech Multiphor 2 electrophoresis system with a multi temp 3 refrigerated bath recirculation unit and an EPS 3501 XL power supply. Pre-cast ampholine gels (Amersham Biosciences, pI range 2.5-10) are loaded with 5 μg of protein. Broad range pI marker standards (Amersham, pI range 3-10, 8 μL) are used to determine relative pI for the Mabs. Electrophoresis is performed at 1500 V, 50 mA for 105 minutes. The gel is fixed using a Sigma fixing solution (5x) diluted with purified water to 1x. Staining is performed overnight at room temperature using Simply Blue stain (Invitrogen). Destaining is carried out with a solution that consisted of 25% ethanol, 8% acetic acid and 67% purified water. Isoelectric points are determined using a Bio-Rad Densitometer relative to calibration curves of the standards. The one or more metrics may further include metrics characterizing stability of the domain under one or more different conditions selected from the group consisting of different pH values, different temperatures, different shear stresses, and different freeze/thaw cycles.

[0088] It will be understood by one skilled in the art that the evaluation of candidate domains may be performed on isolated domains or alternatively, each domain may be evaluated in the context of the intact multidomain protein. The latter approach is particularly useful when a plurality of multidomain proteins differ from each other in a single domain. It will be further understood, that an intact multidomain protein comprising a modified domain may be evaluated to determine the effect of a modified domain on one or more metrics of the invention representing one or more therapeutic and/or formulation and/or manufacturing characteristics. It will also be understood by one skilled in the art that the selected domains, whether evaluated as isolated domains or in the context of an intact multidomain protein, may be incorporated into multidomain proteins. For example, a Fab fragment evaluated and selected in the context of an intact antibody may be utilized in the generation of a chimeric protein comprising a toxin. As used herein, the term "evaluate candidate domains" and grammatical variations thereof, specifically encompass the evaluation of both isolated candidate domains and the evaluation of candidate domains in the context of an intact multidomain protein or fragment thereof.

[0089] Thus, the invention provides a method of screening a plurality of multidomain proteins to identify one or more multidomain proteins that have both the desired biological activities and the desired therapeutic and/or formulation characteristics comprising (a) evaluating for each of a plurality of different multidomain proteins one or more metrics representing one or more therapeutic and/or formulation and/or manufacturing characteristics

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of candidate domains of the multidomain proteins, wherein the plurality of different multidomain proteins exhibits a biological activity above a predetermined threshold level; and (b) selecting one or more multidomain proteins from the plurality based on the metrics of the candidate domains. In one embodiment, the method further comprises generating the population of different multidomain proteins. In certain embodiments, the multidomain protein is an antibody. In other embodiments the modified domain is an antigen binding domain. In still other embodiments, the modified domain is an Fc domain or fragment thereof.

[0090] The invention also provides a method for engineering a multidomain protein for preferred therapeutic and/or formulation properties. In one embodiment, the method comprises engineering one or more domains to improve the protein's formulation characteristics. In another embodiment, the method comprises engineering one or more domains to improve the multidomain protein's therapeutic characteristics. In still another embodiment, the method comprises engineering one or more domains to improve both the multidomain protein's therapeutic and formulation characteristics. In specific embodiments, the engineered domain exhibits improved therapeutic and/or formulation characteristics without reducing significantly the protein's pharmacological characteristics. In another specific embodiment, the engineered domain exhibits improved therapeutic and/or formulation characteristics without substantially affecting the protein's pharmacological characteristics. Accordingly, the invention also provides engineered multidomain proteins having preferred therapeutic and/or formulation properties. In certain embodiments, the engineered multidomain proteins having preferred therapeutic and/or formulation properties comprise a modified domain. In specific embodiment, the engineered multidomain proteins having preferred therapeutic and/or formulation properties comprise a modified antibody domain (*e.g.*, antigen binding domain, Fc domain).

[0091] In a specific embodiment, the invention provides a method of engineering a multidomain protein to have improved stability. In certain embodiments, the multidomain protein comprises a domain which either has a low Tm or contributes to the multidomain protein having a low Tm. In one embodiment, a domain of a multidomain protein is modified by substituting one or more amino acid residues in the domain such that the stability of the domain is increased. In another embodiment, a domain is modified such that its Tm value is increased. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b)

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determining the Tm of the modified domain; and (c) classifying a multidomain protein containing the modified domain as having improved stability if the modified domain is determined to have a higher Tm in step (b). In some embodiments a domain is modified such that it has a Tm greater than the Tm of the domain prior to modification. In certain embodiments, the Tm of the modified domain is increased by at least 2°C, at least 4°C, at least 6°C, at least 8°C, at least 10°C, at least 15°C, at least 20°C, at least 25°C, at least 30°C, or least 40°C. In some embodiments, a domain is engineered such that it has a Tm greater than a predetermined threshold value. In certain embodiments, the predetermined Tm threshold value is at least 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C, 105°C, 110°C, 115°C, or 120°C. In certain embodiments, the multidomain protein is an antibody. In other embodiments said modified domain is an antigen binding domain. In still other embodiments, said modified domain is an Fc domain or fragment thereof.

[0092] As described above, the Tm of a domain may be evaluated in an intact multidomain protein. Accordingly, the present invention provides method of engineering a multidomain protein to have improved stability in which the Tm of intact multidomain protein comprising a modified domain is determined. Optionally, or alternatively, the method of engineering a multidomain protein to have improved stability comprises (a) modifying the domain by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the Tm of the multidomain protein containing the modified domain; and (c) classifying a multidomain protein containing the modified domain based on the Tm of the multidomain protein containing the modified domain determined in step (b).

[0093] The non-specific toxicity and/or biodistribution and/or solubility and/or viscosity of a protein may be optimized by altering the number and location of ionizable residues in the protein to adjust the pI. For example the pI of a polypeptide can be manipulated by making the appropriate amino acid substitutions. For example, at each amino acid residue of a protein, there are several possible changes that can be made: charged residues can be changed to uncharged residues or residues with the opposite charge and uncharged residues can be changed to residues with either a positive or negative charge. The maximum change in charge that can occur is where a charged amino acid is substituted with another amino acid of opposite charge (*e.g.*, by substituting an aspartic acid for a lysine or arginine). Replacing a charged residue with an uncharged residue (*e.g.*, by substituting a charged amino acid such as a lysine, for an uncharged residue such as alanine) serves will

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remove a charge but not add opposite charge and thus will result in a smaller pI change. The acidic amino acids aspartic acid (D) and glutamic acid (E) are deprotonated at or near physiological pH and carry a negative charge. The basic amino acids lysine (K) and arginine (R) are protonated at or near physiological pH and carry a positive charge. Substitutions of D and E with either K or R, or vice versa, are likely to have the greatest impact on pI. Specific amino acid substitutions are described below and detailed in Example 4.

[0094] Without wishing to be bound by any particular theory, amino acid substitutions of a protein that result in changes of the pI of said protein may improve non-specific toxicity and/or biodistribution and/or solubility and/or the viscosity of the protein. One skilled in the art would be able to determine amino acid substitutions that is most appropriate for a particular protein to achieve a desired pI. The pI of a protein may be determined by a variety of methods including but not limited to isoelectric focusing. It can also be estimated using any one of the various computer algorithms (see for example Bjellqvist *et al.*, 1993, *Electrophoresis* 14:1023). In certain embodiments, the invention provides engineered multidomain proteins that have improved non-specific toxicity and/or biodistribution and/or solubility and/or lower viscosity. In a specific embodiment, the engineered multidomain proteins that have improved non-specific toxicity and/or biodistribution solubility and/or lower viscosity comprise a modified domain having different pI than the same domain prior to modification.

[0095] In a specific embodiment, the invention provides a method of engineering a multidomain protein to have improved solubility and/or lower viscosity. In certain embodiments, the multidomain protein comprises a domain which either has a low pI or contributes to the multidomain protein having a low pI. The method comprises (a) modifying a domain of the multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying a multidomain protein containing the modified domain as having improved solubility and/or viscosity if the modified domain is determined to have a higher pI in step (b). In one embodiment a domain is engineered such that it has a pI greater than the pI of the domain prior to modification. In some specific embodiments, the pI of the modified domain is increased by at least 0.5, at least 1.0, at least 2.0, at least 3.0, at least 4.0, at least 5.0, or at least 10.0. In one embodiment, a domain is engineered such that it has a pI greater than a predetermined threshold value. In some specific embodiments, the predetermined pI threshold value is about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0.

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[0096] In certain embodiments, the invention provides engineered multidomain proteins that have improved solubility and/or lower viscosity. In a specific embodiment, the engineered multidomain proteins that have improved solubility and/or lower viscosity comprise a modified domain having a higher pI than the same domain prior to modification. In certain embodiments, the multidomain protein is an antibody. In other embodiments said modified domain is an antigen binding domain. In still other embodiments, said modified domain is an Fc domain or fragment thereof.

[0097] In other specific embodiments, the invention provides a method of engineering a multidomain protein to have reduced non-specific toxicity. In certain embodiments, the multidomain protein comprises a domain which either has a pI or contributes to the multidomain protein having a pI which results in non-specific toxicity. The method comprises (a) modifying a domain of a multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying the multidomain protein containing the modified domain as having reduced non-specific toxicity if the modified domain is determined to have a lower pI in step (b). In one embodiment a domain is engineered such that it has a pI less than the pI of the domain prior to modification. In some specific embodiments, the pI of the modified domain is decreased by at least 0.5, at least 1.0, at least 2.0, at least 3.0, at least 4.0, at least 5.0, or at least 10.0. In one embodiment, a domain is engineered such that it has a pI less than a predetermined threshold value. In some specific embodiments, the predetermined pI threshold value is about 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5 or 5.0.

[0098] In certain embodiments, the invention provides engineered multidomain proteins that have reduced non-specific toxicity. In a specific embodiment, the engineered multidomain proteins that have reduced non-specific toxicity comprises a modified domain having a lower pI than the same domain prior to modification. In certain embodiments, the multidomain protein is an antibody. In other embodiments said modified domain is an antigen binding domain. In still other embodiments, said modified domain is an Fc domain or fragment thereof.

[0099] In additional embodiments, the invention provides a method of engineering a multidomain protein to have a specific biodistribution (*e.g.*, intracellular, extravascular, extracellular). In certain embodiments, the multidomain protein comprises a domain which either has a pI or contributes to the multidomain protein having a pI which determines the biodistribution. The method comprises (a) modifying a domain of a multidomain protein by

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replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the modified domain; and (c) classifying a multidomain protein containing the modified domain as having reduced intracellular localization if the modified domain is determined to have a lower pI in step (b) or as having increased intracellular and/or extravascular localization if the modified domain is determined to have a higher pI in step (b). In one embodiment a domain is modified such that it has a pI greater than the pI of the domain prior to modification. In some specific embodiments, the pI of the modified domain is increased by at least 0.5, at least 1.0, at least 2.0, at least 3.0, at least 4.0, at least 5.0, or at least 10.0. In one embodiment, a domain is modified such that it has a pI greater than a predetermined threshold value. In another embodiment a domain is modified such that it has a pI less than the pI of the domain prior to modification. In some specific embodiments, the pI of the modified domain is decreased by at least 0.5, at least 1.0, at least 2.0, at least 3.0, at least 4.0, at least 5.0, or at least 10.0. In another embodiment, a domain is engineered such that it has a pI less than a predetermined threshold value. In some specific embodiments, the predetermined pI threshold value is about 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5 or 5.0.

[0100] In certain embodiments, the invention provides engineered multidomain proteins that have increased intracellular and/or extravascular localization. In a specific embodiment, the engineered multidomain proteins that have increased intracellular and/or extravascular localization comprise a modified domain having a higher pI than the same domain prior to modification. In other embodiments, the invention provides engineered multidomain proteins that have reduced intracellular localization. In a specific embodiment, the engineered multidomain proteins that have reduced intracellular localization comprise a modified domain having a lower pI than the same domain prior to modification. In certain embodiments, the multidomain protein is an antibody. In other embodiments said modified domain is an antigen binding domain. In still other embodiments, said modified domain is an Fc domain or fragment thereof.

[0101] As described above, the pI of a domain may be evaluated in an intact multidomain protein. Accordingly, the present invention provides methods of engineering a multidomain protein to have improved non-specific toxicity and/or biodistribution solubility and/or lower viscosity in which the pI of intact multidomain protein comprising a modified domain is determined. Optionally, or alternatively, the methods of engineering a multidomain protein to have improved non-specific toxicity and/or biodistribution solubility and/or lower viscosity described above comprise: (a) modifying a domain of a multidomain

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protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) determining the pI of the multidomain protein containing the modified domain; and (c) classifying a multidomain protein containing the modified domain based on the pI of the multidomain protein containing the modified domain determined in step (b).

[0102] In certain embodiments, the modified Fc domain comprises a replacement at one or more amino acid residues selected from the group consisting of K338, A339, K340, G341, Q342, R344, E345, R355, E356, E357, M358, T359, K360, N361, Q362, L365, T366, K370, N390, Y391, K392, T393, T394, V397, L398, D399, S400, D401, F405, K409, L410, D413 and K414 as numbered by the EU index as set forth in Kabat. In specific embodiments, the modified Fc domain comprises one or more amino acid replacements from the group consisting of K338 to any uncharged residue (*e.g.*, Q but generally not P or C), K338D, K338E, A339D, A339E, A339K, A339R, K340 to any uncharged residue (*e.g.*, Q but generally not P or C), K340D, K340E, G314D, G314E, G314K, G314R, Q342D, Q342E, Q342K, Q342R, R344 to any uncharged residue (*e.g.*, Q but generally not P or C), R344D, R344E, E345 to any uncharged residue (*e.g.*, Q but generally not P or C), E345K, E345R, R355 to any uncharged residue (*e.g.*, Q but generally not P or C), R355D, R355E, E356 to any uncharged residue (*e.g.*, Q but generally not P or C), E356K, E356R, E357 to any uncharged residue (*e.g.*, Q but generally not P or C), E357K, E357R, M358D, M358E, M358K, M358R, T359D, T359E, T359K, T359R, K360 to any uncharged residue (*e.g.*, Q but generally not P or C), K360D, K360E, N361D, N361E, N361K, N361R, Q362D, Q362E, Q362K, Q362R, L365D, L365E, L365K, L365R, T366D, T366E, T366K, T366R, K370 to any uncharged residue (*e.g.*, Q but generally not P or C), K370D, K370E, N390D, N390E, N390K, N390R, Y391D, Y391E, Y391K, Y391R, K392 to any uncharged residue (*e.g.*, Q but generally not P or C), K392D, K392E, T393D, T393E, T393K, T393R, T394D, T394E, T394K, T394R, V397D, V397E, V397K, V397R, L398D, L398E, L398K, L398R, D399 to any uncharged residue (*e.g.*, Q but generally not P or C), D399K, D399R, S400D, S400E, S400K, S400R, D401 to any uncharged residue (*e.g.*, Q but generally not P or C), D401K, D401R, F405D, F405E, F405K, F405R, K409 to any uncharged residue (*e.g.*, Q but generally not P or C), K409D, K409E, L410D, L410E, L410K, L410R, D413 to any uncharged residue (*e.g.*, Q but generally not P or C), D413K, D413R, K414 to any uncharged residue (*e.g.*, Q but generally not P or C), K414D and K414E as numbered by the EU index as set forth in Kabat.

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[0103] In certain embodiments, the engineered Fc domain with a higher pI comprises a replacement at one or more amino acid residues selected from the group consisting of A339, G341, Q342, E345, E356, E357, M358, T359, N361, Q362, L365, T366, N390, Y391, T393, T394, V397, L398, D399, S400, D401, F405, L410 and D413 as numbered by the EU index as set forth in Kabat. In specific embodiments, the engineered Fc domain with a higher pI comprises one or more amino acid replacements selected from the group consisting of A339K, A339R, G314K, G314R, Q342K, Q342R, E345 to any uncharged residue (*e.g.*, Q but generally not P or C), E345K, E345R, E356 to any uncharged residue (*e.g.*, Q but generally not P or C), E356K, E356R, E357 to any uncharged residue (*e.g.*, Q but generally not P or C), E357K, E357R, M358K, M358R, T359K, T359R, N361K, N361R, Q362K, Q362R, L365K, L365R, T366K, T366R, N390K, N390R, Y391K, Y391R, T393K, T393R, T394K, T394R, V397K, V397R, L398K, L398R, D399 to any uncharged residue (*e.g.*, Q but generally not P or C), D399K, D399R, S400K, S400R, D401 to any uncharged residue (*e.g.*, Q but generally not P or C), D401K, D401R, F405K, F405R, L410K, L410R, D413 to any uncharged residue (*e.g.*, Q but generally not P or C), D413K and D413R as numbered by the EU index as set forth in Kabat.

[0104] In certain embodiments, the engineered Fc domain with a lower pI comprises a replacement at one or more amino acid residues selected from the group consisting of K338, A339, K340, G341, Q342, R344, R355, M358, T359, K360, N361, Q362, L365, T366, K370, N390, Y391, K392, T393, T394, V397, L398, S400, F405, K409, L410, and K414 as numbered by the EU index as set forth in Kabat. In specific embodiments, the engineered Fc domain with a lower pI comprises one or more amino acid replacements selected from the group consisting of K338 to any uncharged residue (*e.g.*, Q but generally not P or C), K338D, K338E, A339D, A339E, K340 to any uncharged residue (*e.g.*, Q but generally not P or C), K340D, K340E, G314D, G314E, Q342D, Q342E, R344 to any uncharged residue (*e.g.*, Q but generally not P or C), R344D, R344E, R355 to any uncharged residue (*e.g.*, Q but generally not P or C), R355D, R355E, M358D, M358E, T359D, T359E, K360 to any uncharged residue (*e.g.*, Q but generally not P or C), K360D, K360E, N361D, N361E, Q362D, Q362E, L365D, L365E, T366D, K370 to any uncharged residue (*e.g.*, Q but generally not P or C), K370D, K370E, N390D, N390E, Y391D, Y391E, K392 to any uncharged residue (*e.g.*, Q but generally not P or C), K392D, K392E, T393D, T393E, T394D, T394E, V397D, V397E, L398D, L398E, S400D, S400E, F405D, F405E, K409 to any uncharged residue (*e.g.*, Q but generally not P or C), K409D, K409E, L410D, L410E, K414 to any uncharged residue (*e.g.*,

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Q but generally not P or C), K414D and K414E as numbered by the EU index as set forth in Kabat.

[0105] It is contemplated that evaluation of candidate domains is generally to be performed under substantially similar conditions. For example, each candidate domain, evaluated as an intact multidomain protein or as an isolated domain thereof, is preferably at a similar or identical concentration and is in a similar or identical buffer. Furthermore, all manipulations (*e.g.*, pipetting, mixing, heating, cooling, etc.) are to be performed in a substantially similar manner for each candidate domain being evaluated whenever possible.

[0106] In another embodiment, candidate domains are further evaluated under one or more particular biochemical conditions, *e.g.*, conditions under which the formulated drug is stored and/or used. For example, pH affects the shelf life of a protein. The composition of the solvent, *e.g.*, water, also affects the shelf life of a protein. Glycerol alters the solvation properties of water to favor the native conformation of proteins. Ligands and cofactors in the solution may also affect the shelf life of a protein. Other biochemical parameters that have been shown to affect protein shelf life are: protein concentration, temperature, glutathione redox buffers (GSH, GSSG), the presence of detergents, and the presence of other additives, such as glycerol, arginine-HCl, polyethylene glycol (PEG), and organic solvents. Thus, the candidate domains can be evaluated under various conditions to gain more insights into the domains formulation characteristics, such pH dependence, ionic strength dependence, concentration of salts of the Hofmeister series, glycerol concentration, sucrose concentration, arginine concentration, dithiothreitol concentration, metal ion concentration, shear stress, and freeze/thaw stress, etc. For example, the stability of the candidate domains can be determined under a plurality of different biochemical conditions, either different levels of a particular type of conditions, such as different pH values, different temperatures, different sucrose concentrations, or a combination of different types and levels of conditions.

[0107] Some exemplary biochemical conditions described below are of particular interest. In some embodiments, the formulation may contain histidine with a concentration ranging from about 1 mM to about 100 mM, about 10 mM to about 50 mM, or about 20 mM to about 30 mM. Histidine can be in the form of L-histidine, D-histidine, or a mixture thereof, but L-histidine is the most preferable. Histidine can be also in the form of hydrates. Histidine may be used in a form of pharmaceutically acceptable salt, such as hydrochloride (*e.g.*, monohydrochloride and dihydrochloride), hydrobromide, sulfate, acetate, etc. The purity of histidine should be at least 98%, or at least 99%, or at least 99.5%.

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[0108] The pH of the formulation should not be equal to the isoelectric point of the particular multidomain protein (*e.g.*, antibody) to be used in the formulation and may range from about 5.0 to about 7, or about 5.5 to about 6.5, or about 5.8 to about 6.2, and or about 6.0.

[0109] In addition to histidine and a multidomain protein (*e.*g., an antibody) , the formulations may further comprise one or more amino acids (*e.g.*, glycine) at a concentration of less than 100 mM, less than 50 mM, less than 3.0 mM, less than 2.0 mM, or less than 1.8 mM, and most preferably 1.6 mM. The amount of amino acid in the formulation should not cause a significant buffering effect so that protein precipitation at its isoelectric point can be avoided. An amino acid may be also used in a form of pharmaceutically acceptable salt, such as hydrochloride, hydrobromide, sulfate, acetate, etc. The purity of the amino acid should be at least 98%, or at least 99%, or 99.5%. In a specific embodiment, glycine is included in the formulations of the present invention.

[0110] Optionally, the formulations may further comprise other excipients, such as saccharides (*e.g.*, sucrose, mannose, trehalose, etc.), polyols (*e.g.*, Tween) and sugar alcohols (*e.g.*, mannitol, sorbitol, etc.). In one embodiment, the other excipient is a saccharide. In a specific embodiment, the saccharide is sucrose, which is at a concentration ranging from between about 1% to about 20%, or about 5% to about 15%, or about 8% to 10%. In another embodiment, the other excipient is a polyol. Preferably, however, the formulations of the present invention do not contain mannitol. In a specific embodiment, the polyol is polysorbate (*e.g.*, Tween 20), which is at a concentration ranging from between about 0.001% to about 1%, or about 0.01 to about 0.1.

[0111] In one embodiment, candidate domains are evaluated by one or more particular physical manipulations. For example candidate domains can be repeatedly subjected to shearing forces such as pumping, shaking, vortexing and the like to determine their ability to withstand shear stress such as those encountered during manufacture and shipping.

[0112] In a specific embodiment, the present invention provides a method for therapeutic antibody development. As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, camelised antibodies, chimeric antibodies and anti-idiotypic (anti-Id) antibodies. The terms "antibody fragment" and "antibody domain" refer to any distinct region of antibody including but not limited to, "antigen binding domains" which bind an epitope, such

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as, Fab fragments, F(ab') fragments, Fv domains, Fd domain, "constant region domains" such as, CH1, hinge domain and Fc domains and any portions thereof, including for example, CH2 and CH3 domains. Single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), and similar recombinantly generated antigen binding domains are also encompassed by the terms "antibody fragment" and "antibody domain". In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, as well as antibody domain fusion proteins *i.e.*, molecules that contain an antigen binding site or other antibody domain, fused to another protein domain including but not limited to non-immunoglobulin proteins, an Fc domain or fragment thereof. As outlined herein, the terms "antibody" and "antibodies" specifically include the Fc variants described herein, full length antibodies and variant Fc-Fusions comprising Fc domains, or fragments thereof, comprising at least one novel amino acid residue described herein fused to an immunologically active fragment of an immunoglobulin or to other proteins as described herein. Such variant Fc fusions include but are not limited to, scFv-Fc fusions, variable region (*e.g.*, VL and VH) –Fc fusions, scFv-scFv-Fc fusions. Immunoglobulin molecules can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass.

[0113] The present invention also specifically encompasses antibodies with multiple specificities (*e.g.*, an antibody with specificity for two or more discrete antigens (reviewed in Cao et al., 2003, *Adv Drug Deliv Rev* 55:171; Hudson et al., 2003, *Nat Med* 1:129)). For example, bispecific antibodies contain two different binding specificities fused together. In the simplest case a bispecific antibody would bind to two adjacent epitopes on a single target antigen, such an antibody would not cross-react with other antigens. Alternatively, bispecific antibodies can bind to two different antigens, such an antibody specifically binds to two different molecules such as a heterologous polypeptide or solid support material, but not to other unrelated molecules (*e.g.*, BSA). *See, e.g.*, PCT publications WO 93/17715; WO 92/08802; WO91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Pat. Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992). While such molecules normally will only bind two antigens (*i.e.* bispecific antibodies), antibodies with additional specificities such as trispecific antibodies are encompassed by the instant invention. Accordingly, the multidomain proteins of the present invention may be antibodies that are monospecific, bispecific, trispecific or of greater multispecificity.

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[0114] It will be understood that Fc domain as used herein includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, the Fc domain comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cγ2 and Cγ3) and the hinge between Cgamma1 (Cγ1) and Cgamma2 (Cγ2). Although the boundaries of the Fc domain may vary, the human IgG heavy chain Fc domain is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, VA). The "EU index as set forth in Kabat" refers to the residue numbering of the human IgG1 EU antibody as described in Kabat et al. *supra*. Fc may refer to this domain in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion protein. An Fc containing protein may be an antibody, Fc fusion, or any protein or protein domain that comprises an Fc domain. The present invention also encompasses proteins comprising variant Fc domains, which are non naturally occurring variants of an Fc. Note: Polymorphisms have been observed at a number of Fc positions, including but not limited to Kabat 270, 272, 312, 315, 356, and 358, and thus slight differences between the presented sequence and sequences in the prior art may exist.

[0115] The method for therapeutic antibody development comprises screening a plurality of candidate antibody domains (*e.g.*, Fab, Fc and Fv) that have high biological activity (*e.g.*, binding affinity to a target antigen (*e.g.*, antigen, Fc-receptor), effector function (*e.g.*, ADCC, CDC)) for one or more metrics characterizing the therapeutic and/or formulation characteristics. In one embodiment, the metrics include one or more parameters characterizing stability of said antibody domain. In another embodiment, the metrics include one or more parameters characterizing solubility, biodistribution or non-specific toxicity of said antibody domain. In a specific embodiment, said one or more parameters characterizing stability of said antibody domain comprise a Tm value of said antibody domain, and said one or more parameters characterizing solubility, biodistribution or non-specific toxicity of said antibody domain comprise a pI value of said antibody domain.

[0116] The present invention also provides a method of generating one or more antibodies for therapeutic uses. In one embodiment, the method of generating one or more antibodies for therapeutic uses, comprises (a) evaluating for each of a plurality of candidate

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antibody domains one or more metrics representing one or more therapeutic and/or formulation and/or manufacturing characteristics of the antibody domain, wherein the plurality of candidate antibody domains exhibits a biological activity above a predetermined threshold level; (b) selecting one or more antibody domains from the plurality based on the metrics; and (c) optionally, constructing an antibody using each antibody domain selected in step (b) and one or more other domains.

[0117] In another embodiment, the method of generating one or more antibodies for therapeutic uses further comprises, before using the selected antibody domain to construct an antibody (prior to step (c)), the steps of (i) evaluating for each of a plurality of candidate other antibody domains one or more metrics; and (ii) selecting another antibody domain from the plurality of candidate other antibody domains based on the metrics.

[0118] In one embodiment, the plurality of candidate antibody domains can be an antigen binding domains (*e.g.*, Fab domains). In such cases, the biological activity may be the antigen binding domain's binding affinity to a target antigen. In certain embodiments, at least some of the antigen binding domains bind different epitopes of the target antigen. In a specific embodiment, the plurality of antigen binding domains are obtained by screening an expression library (*e.g.*, a phage display library) with the target antigen. In another specific embodiment, the plurality of antigen binding domains are obtained by digesting a plurality of monoclonal antibodies. Each selected antigen binding domain is then combined with another protein domain (*e.g.*, Fc domain) to generate one or more multidomain proteins. In a specific embodiment, each selected antigen binding domain is combined with another antigen binding domain which may bind to the same or a different target antigen. In another specific embodiment, each selected antigen binding domain is combined with an antibody constant domain.

[0119] In another embodiment, the plurality of candidate antibody domains can be Fc domains. In such cases, the biological activity may be the Fc domain's binding affinity to constant region domain receptors an/or ligands (*e.g.*, FcRn, C1q, FcγRs) and/or ability to mediate effector functions (*e.g.*, ADCC, CDC). In a specific embodiment, the plurality of Fc domains are obtained by screening an expression library (*e.g.*, a phage display library) with one or more of the constant region domain receptors an/or ligands. Each selected Fc is then combined with another protein domain (*e.g.*, a Fab domain, a cellular receptor domain) to generate one or more multidomain proteins.

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[0120] The methods described above for screening and/or engineering protein domains for their non-specific toxicity, biodistribution, solubility and thermal stability can be used. One or more antibody domains having reduced non-specific toxicity and/or desired biodistribution and/or high solubility and/or thermal stability are selected and used for constructing the full antibodies by combining them with the appropriate domain(s) to generate a full antibody. In one embodiment, one or more candidate antibody domains that have a Tm value higher than at least 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C, 105°C, 110°C, 115°C, or 120°C are selected for construction of the full antibody. In another embodiment, one or more candidate antibody domains that have a pI value higher than about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0 are selected for construction of the full antibody domain containing multidomain protein. In another embodiment, one or more candidate antibody domains that have a pI value less than about 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5 or 5.0 are selected for construction of the full antibody domain containing multidomain protein

[0121] In a specific embodiment, a plurality of antigen binding domains (*e.g.*, Fab, scFv, etc.) that bind a given target antigen with an affinity above a chosen threshold level are obtained by affinity screening of an expression library (*e.g.*, a phage display library). One or more metrics characterizing the antigen binding domains' therapeutic and/or formulation properties are then evaluated for each of the antigen binding domains. The plurality of antigen binding domains are ranked according to the one or more metrics. In one embodiment, the plurality of antigen binding domains are ranked according to their Tm values, and one or more antigen binding domains are selected from the top of the ranked list. In another embodiment, the plurality of antigen binding domains are ranked according to their pI values, and one or more antigen binding domains are selected from the top of the ranked list. In still another embodiment, the plurality of antigen binding domains are ranked according to a combined Tm and pI rank, and one or more antigen binding domains are selected from the top of the ranked list. The selected antigen binding domains are then used for construction of the full antigen binding domain containing multidomain proteins (*e.g.*, antibodies, diabodies, etc.).

[0122] In another specific embodiment, a plurality of antibody constant region domains (*e.g.*, Fc, CH2, CH3, etc) are evaluated for one or more metrics characterizing the constant region domains' therapeutic and/or formulation properties (*e.g.*, solubility and thermal stability). The plurality of antigen binding domains are ranked according to the one

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or more metrics. In one embodiment, the plurality of constant region domains are ranked according to their Tm values, and one or more constant region domains are selected from the top of the ranked list. In another embodiment, the plurality of constant region domains are ranked according to their pI values, and one or more constant region domains are selected from the top of the ranked list. In still another embodiment, the plurality of constant region domains are ranked according to a combined Tm and pI rank, and one or more constant region domains are selected from the top of the ranked list. The selected constant region domains are then used for construction of the full antigen binding domain containing multidomain proteins (*e.g.*, antibodies, diabodies, etc.). In certain embodiments, one or more candidate antibody constant region domains that have a Tm value higher than at least 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C, 105°C, 110°C, 115°C, or 120°C are selected for construction of the full antibody constant region domain containing multidomain protein (*e.g.*, antibody). In other embodiments, one or more candidate antibody constant region domains that have a pI value higher than about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0 are selected for construction of the full antibody constant region domain containing multidomain protein (*e.g.*, antibody, Fc-fusion protein, etc.). In still other embodiments, one or more candidate antibody constant region domains that have a pI value lower than about 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, or 5.0 are selected for construction of the full antibody constant region domain containing multidomain protein.

[0123] In a specific embodiment, the invention provides a method for engineering an antibody for preferred therapeutic and/or formulation properties. In one embodiment, the method comprises engineering the antigen binding (*e.g.*, Fab) and/or constant region (*e.g.*, Fc) domains to improve the protein's therapeutic and/or formulation characteristics. The method comprises making one or more modification (*e.g.*, amino acid substitutions), the modifications are such that they improve one or more of the characteristics of said antibody.

[0124] In addition to binding antigen, antibodies are known to bind to a number of ligands via their constant region domain including, Fc receptors (*e.g.*, FcRn, FcγRs) and the complement protein C1q. The binding interactions of the constant region domain are essential for a variety of effector functions and downstream signaling events such as antibody dependent cell-mediated cytotoxicity (ADCC) activity and complement dependent cytotoxicity (CDC). In certain embodiments, the engineered antibody exhibits improved therapeutic and/or formulation characteristics without reducing significantly the antibody's pharmacological characteristics, *e.g.*, the antibody's binding specificity, binding affinity

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and/or avidity to its target, or the antibody's effector functions, *e.g.*, Fc-receptor (FcR) and/or C1q binding, antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and/or serum half life. In another embodiment, the engineered antibody exhibits improved therapeutic and/or formulation characteristics and improved pharmacological characteristics, *e.g.*, the antibody's binding specificity, binding affinity and/or avidity to its target, or the antibody's Fc effector functions, *e.g.*, FcR binding, ADCC, CDC, and/or serum half life.

[0125] "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Specific high-affinity IgG antibodies directed to the surface of target cells "arm" the cytotoxic cells and are absolutely required for such killing. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement. It is contemplated that, in addition to antibodies, other proteins comprising Fc domain, specifically Fc fusion proteins, having the capacity to bind specifically to an antigen-bearing target cell will be able to effect cell-mediated cytotoxicity. For simplicity, the cell-mediated cytotoxicity resulting from the activity of an Fc fusion protein is also referred to herein as ADCC activity.

[0126] The ability of any particular protein comprising an Fc to mediate lysis of the target cell by ADCC can be assayed. To assess ADCC activity a protein comprising an Fc is added to target cells in combination with immune effector cells, which may be activated by the antigen antibody complexes resulting in cytolysis of the target cell. Cytolysis is generally detected by the release of label (*e.g.* radioactive substrates, fluorescent dyes or natural intracellular proteins) from the lysed cells. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Specific examples of *in vitro* ADCC assays are described in Wisecarver et al., 1985 79:277-282; Bruggemann et al., 1987, *J Exp Med* 166:1351-1361; Wilkinson et al., 2001, *J Immunol Methods* 258:183-191; Patel et al., 1995 *J Immunol Methods* 184:29-38 and herein (see Example 3). Alternatively, or additionally, ADCC activity of the protein comprising an Fc may be assessed *in vivo, e.g.,* in an animal model such as that disclosed in Clynes et al., 1998, PNAS *USA* 95:652-656.

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[0127] "Complement dependent cytotoxicity" and "CDC" refer to the lysing of a target cell in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule, an antibody for example, complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro et al., 1996, *J. Immunol. Methods,* 202:163, may be performed.

[0128] In one embodiment, the pI of an engineered antibody binding domain is between pH 5.0 and pH 10.0. In one embodiment, substitutions resulting in alterations in the pI of the antigen binding domain will not significantly diminish its binding affinity for an antigen. In one embodiment, the pI of an engineered antibody constant region domain is between pH 5.0 and pH 10.0. In still another embodiment, substitutions resulting in alterations in the pI of the constant region domain will not significantly diminish its effector binding and/or function. It is also contemplated that substitutions resulting in alterations in the pI in an antibody domain may be selected such that both the pI and other pharmacological characteristics of the antibody domain, *e.g.*, the antibody's binding specificity, binding affinity and/or avidity to its target, or the antibody's Fc effector functions are improved. The inventors have found that certain modifications of the hinge region do not change the pI and Tm of the antibody significantly. Thus, in one embodiment, the invention provides a method for engineering an antibody to improve the antibody's biological activity without reducing the antibody's therapeutic and/or formulation properties.

[0129] In one embodiment, the modifications of an antibody domain as described herein may be combined with known modifications of the Fc domain such as those disclosed in Duncan et al, 1988, *Nature* 332:563-564; Lund *et al.*, 1991, *J. Immunol* 147:2657-2662; Lund et al, 1992, *Mol Immunol* 29:53-59; Alegre et al, 1994, *Transplantation* 57:1537-1543; Hutchins *et al.*, 1995, *Proc Natl. Acad Sci U S A* 92:11980-11984; Jefferis et al, 1995, *Immunol Lett.* 44:111-117; Lund *et al.*, 1995, *Faseb J* 9:115-119; Jefferis et al, 1996, *Immunol Lett* 54:101-104; Lund et al, 1996, *Immunol* 157:4963-4969; Armour *et al.*, 1999, *Eur J Immunol* 29:2613-2624; Idusogie et al, 2000, *J Immunol* 164:4178-4184; Reddy et al, 2000, *J Immunol* 164:1925-1933; Xu *et al.*, 2000, *Cell Immunol* 200:16-26; Idusogie et al, 2001, *J Immunol* 166:2571-2575; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Jefferis et al, 2002, *Immunol Lett* 82:57-65; Presta *et al.*, 2002, *Biochem Soc Trans* 30:487-490); U.S. Pat. Nos. 5,624,821; 5,885,573; 5,677,425; 6,165,745; 6,277,375; 5,869,046; 6,121,022; 5,624,821; 5,648,260; 6,528,624; 6,194,551; 6,737,056; 6,821,505; 6,277,375; U.S. Patent

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Publication No. 2004/0002587; PCT Publication Nos. WO 00/42072 and WO 99/58572; WO 94/29351; WO 02/060919; WO 04/029207; WO 04/099249; WO 04/063351. Also encompassed by the present invention are Fc domains which comprise deletions, additions and/or modifications. Still other modifications/substitutions/additions/deletions of the Fc domain will be readily apparent to one skilled in the art.

[0130] In one embodiment, the antibodies may be engineered to include modifications in the Fc domain, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity, without reducing the antibodies' pI and Tm. Furthermore, an antibody may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody.

[0131] In one embodiment, the amino acid sequence of the Fc domain is modified by deleting, adding and/or substituting at least amino acid residue to alter one or more of the functional properties of the antibody described above. This approach is described further in Duncan et al, 1988, *Nature* 332:563-564; Lund *et al.*, 1991, *J. Immunol* 147:2657-2662; Lund et al, 1992, *Mol Immunol* 29:53-59; Alegre et al, 1994, *Transplantation* 57:1537-1543; Hutchins *et al.*, 1995, *Proc Natl. Acad Sci U S A* 92:11980-11984; Jefferis et al, 1995, *Immunol Lett.* 44:111-117; Lund *et al.*, 1995, *Faseb J* 9:115-119; Jefferis et al, 1996, *Immunol Lett* 54:101-104; Lund et al, 1996, *J Immunol* 157:4963-4969; Armour *et al.*, 1999, *Eur J Immunol* 29:2613-2624; Idusogie et al, 2000, *J Immunol* 164:4178-4184; Reddy et al, 2000, *J Immunol* 164:1925-1933; Xu *et al.*, 2000, *Cell Immunol* 200:16-26; Idusogie et al, 2001, *J Immunol* 166:2571-2575; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Jefferis et al, 2002, *Immunol Lett* 82:57-65; Presta *et al.*, 2002, *Biochem Soc Trans* 30:487-490); U.S. Patent Nos. 5,624,821; 5,885,573; 5,677,425; 6,165,745; 6,277,375; 5,869,046; 6,121,022; 5,624,821; 5,648,260; 6,194,551; 6,737,056 U.S. Patent Application Nos. 10/370,749 and PCT Publications WO 94/2935; WO 99/58572; WO 00/42072; WO 04/029207.

[0132] In still another embodiment, the glycosylation of antibodies is modified. For example, an aglycoslated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for a target antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region

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framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Patent Nos. 5,714,350 and 6,350,861.

[0133] Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNAc structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be engineered by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. See, for example, Shields, R.L. *et al.* (2002) *J. Biol. Chem.* 277:26733-26740; Umana *et al.* (1999) *Nat. Biotech.* 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342. Additional, methods for generating engineered glycoforms are known in the art, and include but are not limited to those described in Davies et al., 20017 *Biotechnol Bioeng* 74:288-294; Shields et al, 2002, *J Biol Chem* 277:26733-26740; Shinkawa et al., 2003, *J Biol Chem* 278:3466-3473) U.S. Pat. No. 6,602,684; U.S. Ser. No. 10/277,370; U.S. Ser. No. 10/113,929; PCT WO 00/61739A1; PCT WO 01/292246A1; PCT WO 02/311140A1; PCT WO 02/30954A1; Potillegent™ technology (Biowa, Inc. Princeton, N.J.); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG, Zurich, Switzerland). See, e.g., WO 00061739; EA01229125; US 20030115614; Okazaki et al., 2004, *JMB*, 336: 1239-49.

[0134] In another embodiment, the antibodies may be engineered to include modifications in the antigen binding domain to alter the therapeutic and/or formulation characteristics of the antibody, without reducing the binding characteristics of the antibody for its antigen. The method comprises making one or more modification (*e.g.*, amino acid substitutions), said modifications are such that they improve said one or more of the therapeutic and/or formulation characteristics of said antibody. One skilled in the art will understand that amino acid substitutions and other modifications of an antibody may alter its binding characteristics (examples of binding characteristics include but are not limited to, binding specificity, equilibrium dissociation constant (K_D), dissociation and association rates (K_{off} and K_{on} respectively), binding affinity and/or avidity) and that certain alterations are more or less desirable. For example a modification that preserves or enhances antigen

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binding would generally be more preferable then one that diminished or altered antigen binding. It will be further understood, that antibodies may be engineered to include modifications in the constant region domain to alter the therapeutic and/or formulation characteristics of the antibody, without reducing the binding characteristics of the constant region to its receptors or ligands (*e.g.*, FcRs, C1q). The binding characteristics of an antibody for a target antigen or constant region domain ligand may be determined by a variety of methods including but not limited it, equilibrium methods (*e.g.*, enzyme-linked immunoabsorbent assay (ELISA) or radioimmunoassay (RIA)), or kinetics (*e.g.*, BIACORE® analysis; see Example 2), for example. Other commonly used methods to examine the binding characteristics of antibodies are described in *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, Harrow *et al.*, 1999 and *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY; Harlow *et al.*, 1989.

[0135] It is well known in the art that the affinity constant (K_a) is defined as k_{on}/k_{off} and that the equilibrium dissociation constant (K_D) is defined as k_{off}/k_{on}. It is generally understood that an antibody with a high K_a is preferable to an antibody with a low K_a and that an antibody with a low K_D is preferable to an antibody with a high K_D. However, in some instances the value of the k_{on} or k_{off} may be more relevant than the value of the K_D. One skilled in the art can determine which kinetic parameter is most important for a given antigen binding domain and application. In one embodiment, the methods of the invention will result in modified antigen binding and/or constant region domains with improved therapeutic and/or formulation characteristics and one or more binding characteristics (*e.g.*, binding specificity, K_D, K_{off}, K_{on}, binding affinity and/or avidity) that are improved by at least 2%, or by at least 5%, or by at least 10%, or by at least 20 %, or by at least 30%, or by at least 40%, or by at least 50%, or by at least 60%, or by at least 70%, or by at least 80% when compared to kinetic parameters of the antigen binding and/or constant region domain without said modification.

[0136] In another embodiment, the methods of the invention will result in modified antigen binding and/or constant region domains that have improved therapeutic and/or formulation characteristics, but do not have substantially diminished binding characteristics. For example, the methods of the invention will generate antigen binding and/or constant region domains that exhibit improved formulation characteristics, but preferably have no reduction in any binding characteristic (*e.g.*, binding specificity, K_D, K_{off}, K_{on}, binding

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affinity and/or avidity), or have one or more antigen binding characteristics that are reduced by less than 1%, or by less than 5%, or by less than 10%, or by less than 20 %, or by less than 30%, or by less than 40%, or by less than 50%, or by less than 60%, or by less than 70%, or by less than 80% when compared to antigen binding of the antibody without said substitution.

[0137] In yet another embodiment, the methods of the invention will result in the selection of antigen binding and/or constant region domains with a binding activity above a predetermined threshold having improved therapeutic and/or formulation characteristics. In certain embodiments, the binding activity is the affinity constant or K_a (k_{on}/k_{off}) of the domain for a particular target/ligand/receptor. In a specific embodiment, the K_a is at least 10^2M^{-1}, at least $5\times10^2M^{-1}$, at least 10^3M^{-1}, at least $5\times10^3M^{-1}$, at least 10^4M^{-1}, at least $5\times10^4M^{-1}$, at least 10^5M^{-1}, at least $5\times10^5M^{-1}$, at least 10^6M^{-1}, at least $5\times10^6M^{-1}$, at least 10^7M^{-1}, at least $5\times10^7M^{-1}$, at least 10^8M^{-1}, at least $5\times10^8M^{-1}$, at least 10^9M^{-1}, at least $5\times10^9M^{-1}$, at least $10^{10}M^{-1}$, at least $5\times10^1M^{-1}$, at least $10^{11}M^{-1}$, at least $5\times10^{11}M^{-1}$, at least $10^{12}M^{-1}$, at least $5\times10^{12}M$, at least $10^{13}M^{-1}$, at least $5\times10^{13}M^{-1}$, at least $10^{14}M^{-1}$, at least $5\times10^{14}M^{-1}$, at least $10^{15}M^{-1}$, or at least $5\times10^{15}M^{-1}$. In other embodiments, the binding activity is the equilibrium dissociation constant (K_D) of the domain for a particular target/ligand/receptor. As described above, one of skill in the art will recognize that a molecule having a higher binding affinity will generally have a lower K_D value. Accordingly, in a specific embodiment the K_D is less than 10^{-5} M, or less than 10^{-6} M, or less than 10^{-7} M, or less than 10^{-8} M, or less than 10^{-9} M, or less than 10^{-10} M, or less than 10^{-11} M, or less than 10^{-12} M, or less than 10^{-13} M.

[0138] In one embodiment, selected or engineered antigen binding and/or constant region domains are then used to construct a full antigen binding and/or antibody constant domain containing multidomain protein (*e.g.*, antibody) using methods known in the art. Such multidomain proteins can then be submitted to formulation development to determine the optimal formulations.

[0139] There are various methods available for assessing the stability of protein drug formulations (*e.g.*, multidomain protein formulations), based on the physical and chemical structures of the proteins (*e.g.*, antibodies or fragments thereof) as well as on their biological activities. For example, to study denaturation of proteins, methods such as charge-transfer absorption, thermal analysis, fluorescence spectroscopy, circular dichroism, NMR, and HPSEC, are available. *See*, for example, Wang *et al.*, 1988, J. of Parenteral Science & Technology 42 (Suppl):S4-S26.

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[0140] The rCGE and HPSEC are the most common and simplest methods to assess the formation of protein aggregates, protein degradation, and protein fragmentation. Accordingly, the stability of the formulations may be assessed by these methods.

[0141] In certain embodiments, the concentration of the multidomain protein formulations is at least 15 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 30 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml, at least 50 mg/ml, at least 55 mg/ml, at least 60 mg/ml, at least 65 mg/ml, at least 70 mg/ml, at least 75 mg/ml, at least 80 mg/ml, at least 85 mg/ml, at least 90 mg/ml, at least 95 mg/ml, at least 100 mg/ml, at least 105 mg/ml, at least 110 mg/ml, at least 115 mg/ml, at least 120 mg/ml, at least 125 mg/ml, at least 130 mg/ml, at least 135 mg/ml, at least 140 mg/ml, at least 150 mg/ml, at least 200 mg/ml, at least 250 mg/ml, or at least 300 mg/ml.

[0142] In certain embodiments, the formulations of the multidomain protein exhibit stability at the temperature ranges of 38°C-42°C for at least 30 days, at least 45 days, or at least 60 days and, in some embodiments, not more than 120 days, of 20°C-24°C for at least 6 months, or at least 1 year, of 2°C-8°C (in particular, at 4°C) for at least 6 months, at least 1 year, at least 2 years, at least 3 years, at least 4 years, or at least 5 years and at -20°C for at least 1 year, at least 2 years, at least 3 years, at least 4 years, or at least 5 years, as assessed by high performance size exclusion chromatography (HPSEC). Namely, the formulations have low to undetectable levels of aggregation and/or fragmentation, as defined herein, after the storage for the defined periods as set forth above. Preferably, no more than 5%, no more than 4%, no more than 3%, no more than 2%, no more than 1%, no more than 0.5% of the protein or protein fragment forms an aggregate as measured by HPSEC, after the storage for the defined periods as set forth above. In specific embodiments, formulations of the multidomain protein exhibit almost no loss in biological activities of the multidomain protein during the prolonged storage under the condition described above, as assessed by various assays including, but not limited to, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay to measure the ability of, for example, an antibody or antibody fragment to specifically bind to a target antigen, by a C3a/C4a assay to measure the complement activating ability of the antibody and by chromium release assay to measure the ADCC activity of the antibody. The formulations retain after the storage for the above-defined periods more than 80%, more than 85%, more than 90%, more than 95%, more than 98%, more than 99%, or more than 99.5% of the initial biological activities of the formulation prior to the storage.

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[0143] For example, the stability of the formulations may be evaluated by HPSEC or rCGE, wherein the percent area of the peaks represents the non-degraded protein. In particular, approximately 250 μg of, for example, the antibody or antibody fragment that specifically binds to a target antigen (approximately 25 μl of a liquid formulation comprising 10 mg/ml said antibody or antibody fragment) is injected onto a TosoH Biosep TSK G3000SWXL column (7.8 mm x 30 cm) fitted with a TSK SW x1 guard column (6.0 mm CX 4.0 cm). The antibody or antibody fragment is eluted isocratically with 0.1 M disodium phosphate containing 0.1 M sodium sulfate and 0.05% sodium azide, at a flow rate of 0.8 to 1.0 ml/min. Eluted protein is detected using UV absorbance at 280 nm. A suitable reference standard is run in the assay as a control, and the results are reported as the area percent of the product monomer peak compared to all other peaks excluding the included volume peak observed at approximately 12 to 14 minutes. Peaks eluting earlier than the monomer peak are recorded as percent aggregate.

[0144] In certain embodiments, the formulations of the multidomain protein exhibit low to undetectable levels of aggregation as measured by HPSEC or rCGE, that is, no more than 5%, no more than 4%, no more than 3%, no more than 2%, no more than 1%, or no more than 0.5% aggregate by weight protein, and low to undetectable levels of fragmentation, that is, 80% or higher, 85% or higher, 90% or higher, 95% or higher, 98% or higher, or 99% or higher, or 99.5% or higher of the total peak area in the peak(s) representing intact protein. In the case of SDS-PAGE, the density or the radioactivity of each band stained or labeled with radioisotope can be measured and the % density or % radioactivity of the band representing non-degraded multidomain protein can be obtained.

[0145] The stability of the formulations can be also assessed by any assays that measure the biological activity of the multidomain protein in the formulation. For example, the biological activities of antibodies include, but are not limited to, antigen-binding activity, complement-activation activity, Fc-receptor binding activity, and so forth. Antigen-binding activity of the antibodies can be measured by any method known to those skilled in the art, including but not limited to ELISA, radioimmunoassay, Western blot, BIAcore, and the like. Complement-activation activity can be measured by a C3a/C4a assay in the system where the antibody which specifically binds to an antigen is reacted in the presence of the complement components with the cells expressing the antigen. Also see Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988). An ELISA based assay, *e.g.*, may be used to compare the ability of an antibody or fragment thereof to

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specifically bind to a target antigen to a suitable reference standard. In this assay, plates are coated with the target antigen and the binding signal of a set concentration of a suitable reference standard is compared to the binding signal of the same concentration of a test antibody or antibody fragment.

[0146] The purity of the multidomain protein formulations may be measured by any method well-known to one of skill in the art such as, *e.g.*, HPSEC. The sterility of the multidomain protein formulations may be assessed by methods well known in the art. For example, sterile soybean-casein digest medium and fluid thioglycollate medium are inoculated with a test protein formulation by filtering a liquid protein formulation through a sterile filter having a nominal porosity of 0.45 μm. When using the Sterisure™ or Steritest™ method, each filter device is aseptically filled with approximately 100 ml of sterile soybean-casein digest medium or fluid thioglycollate medium. When using the conventional method, the challenged filter is aseptically transferred to 100 ml of sterile soybean-casein digest medium or fluid thioglycollate medium. The media are incubated at appropriate temperatures and observed three times over a 14 day period for evidence of bacterial or fungal growth.

[0147] The methods described above can be used to generate antibody therapeutics targeting any infectious organisms, including but not limited to any viruses and bacteria.

[0148] The methods described above can also be used to generate antibody therapeutics targeting other antigens, including but not limited to 17-1A, α4β7 integrin receptor, AFP, CBL, CD11, CD11a, CD14, CD147, CD18, CD20, CD22, CD23, CD25, CD3, CD33, CD4, CD40, CD40L, CD44, CD52, CD64 (FcR), CD80, CEA, Complement (C5), CTLA-4, EGF receptor, Ep-CAM, E-selectin, Factor VII, FcRI receptors, Gamma Interferon, GD2-ganglioside, gp IIIb/IIIa, gp72, HER-2, HLA-DR 10 beta, HLA-DR antigen, ICAM-3, IgE, IL-4, IL5, IL-5, IL-8, inhibitor of P38MAP kinase, inosine monophosphate dehydrogenase, ganglioside GD3, MUC-1, nuC242, PEM antigen, SK-1 antigen, TNF alpha, VEGF, VEGF-receptor, and VLA-4.

[0149] In certain specific embodiments, the methods described above can also be used to generate antibody therapeutics that specifically binds to an antigen of RSV, an antigen of human metapneumovirus (hMPV), integrin $\alpha_v\beta_3$, CD2, CD19, EphA2, EphA4, or IL-9. Additional nonlimiting therapeutic targets are described in section 5.3 below.

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5.2. Methods For Evaluating And/Or Improving The Shelf Life Of A Formulations Comprising A Multidomain Protein

[0150] The invention also provides a method for evaluating the shelf-life of a multidomain protein formulation. The methods of the invention involves evaluating the thermal denaturation and/or renaturation behavior of a multidomain protein in a liquid preparation, which comprises a multidomain protein of interest having a domain the unfolding of which leads to aggregation of the multidomain protein in the liquid protein preparation. In one embodiment, the denaturation/renaturation behavior of the liquid protein preparation is determined by measuring a thermal denaturation/renaturation curve. The method then provides an indicator for the shelf life of the preparation based on the thermal denaturation and/or renaturation curve. For example, if the domain does not refold upon cooling or denatures at a low temperature, the multidomain protein is likely to have a short shelf life.

[0151] The invention also provides a method of evaluating long term stability of a multidomain protein in a solution, wherein the long term stability is defined as less than 5%, less than 10%, or less than 20% aggregation upon storage for a predetermined period of time at a predetermined temperature. The method comprises (a) providing a solution of a multidomain protein; (b) denaturing one or more domains of the multidomain protein by heating the solution of the multidomain protein; (c) determining if the domains refold upon cooling; and (d) classifying the multidomain protein as having long term stability in the solution if the domains are determined to refold in step (c). It is contemplated that the stability of a multidomain protein may be evaluated in a number of different solutions thereby identifying a particular solution useful for stabilizing a multidomain protein. Alternatively, or optionally, the method may be utilized to compare several different multidomain proteins having a desired biological activity above a predetermined threshold level, wherein each multidomain protein is formulated in a solution having identical components with the exception of the multidomain protein.

[0152] It is contemplated that a multidomain protein may comprise a domain the unfolding of which causes aggregation of the multidomain protein. Accordingly, the invention also provides a method for identifying a domain in a multidomain protein, which domain is responsible for aggregation of the protein. In one embodiment, the method of identifying one or more domain in a multidomain protein, which is responsible for aggregation comprises (a) denaturing one or more domains of the multidomain protein by

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heating said solution; (b) determining if one or more of said domains refold upon cooling; and (c) identifying one or more domains that do not refold upon cooling in step (b), thereby identifying a domain or domains, the unfolding of which causes aggregation of said multidomain protein in said solution.

[0153] The invention also provides a method of engineering a multidomain protein to have improved long term stability in a solution, wherein said long term stability is defined as less than 5%, less than 10%, or less than 20% aggregation upon storage for a predetermined period of time at a predetermined temperature. The method of engineering a multidomain protein to have improved long term stability in a solution comprises (a) modifying a domain of the multidomain protein by replacing one or more amino acid residues in the domain to generated a modified domain; (b) denaturing the modified domain by heating a solution of a multidomain protein comprising the modified domain; (c) determining if said modified domain in the multidomain protein refolds upon cooling; and (d) classifying the multidomain protein containing the modified domain as having improved long term stability if the modified domain is determined to refold in step (c). In one embodiment, the multidomain protein is an antibody. It is contemplated that steps (a) to (d) may be performed on an isolated domain. For example, a Fab domain, the unfolding of which causes aggregation of an antibody, may be isolated, modified denatured, renatured and classified. In one embodiment, the identified domain having improved long term stability is used in the generation of a multidomain protein. In one embodiment, the multidomain protein is an antibody. In another embodiment, the domain is an antigen binding domain. In yet another embodiment, the domain is a constant region domain. In certain embodiments, the multidomain protein comprises a domain the unfolding of which causes aggregation of the multidomain protein in the solution.

[0154] The invention further provides a method of screening for a multidomain protein which has improved long term stability in a solution, wherein the long term stability is defined as less than 5%, less than 10%, or less than 20% aggregation upon storage for a predetermined period of time at a predetermined temperature. In certain embodiments, a multidomain protein comprises a domain the unfolding of which causes aggregation of the multidomain protein in a solution. In one embodiment, the method comprises (a) denaturing respectively two or more multidomain proteins that are members of a population multidomain proteins, wherein each multidomain proteins comprises a different modified domain, the modified domain having one or more amino acid residues substituted, wherein the denaturing

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is accomplished by heating a solution of one of the multidomain proteins comprising the modified domain; (b) determining if the different modified domains in each multidomain protein denatured in step (a) refolds upon cooling; and (c) identifying the multidomain proteins containing a modified domain that is determined to refold in step (b) as having improved long term stability. In one embodiment, the method further comprises generating the population of different multidomain proteins. It is contemplated that steps (a) to (c) may be performed on isolated domains. For example, a Fab domain, the unfolding of which causes aggregation of an antibody, may be isolated and modified to generate a population of different modified domains two or more of which may then be screened by the method. In one embodiment, the identified domain having improved long term stability is used in the generation of a multidomain protein. In a specific embodiment the multidomain protein is an antibody. In one embodiment, the domain is an antigen binding domain (e.g., a Fab domain). In another embodiment, the domain is a constant region domain.

[0155] In certain embodiments, the denaturation and renaturation (i.e., refolding upon cooling) steps are carried out by generating a thermal denaturation/renaturation curve, e.g., using differential scanning calorimetry. The magnitude of the change in a physical property associated with the thermal denaturation of the multidomain protein as a result of heating is measured over a range of temperatures to generate a thermal denaturation/renaturation curve. It is contemplated that the temperature range encompasses the desired storage temperature range.

[0156] As used herein, a "thermal denaturation/renaturation curve" is a plot of a change in a physical property associated with the denaturation or renaturation of a protein as a function of temperature. See, for example, Davidson et al., Nature Structure Biology 2:859 (1995); Clegg, R. M. et al., Proc. Natl. Acad. Sci. U.S.A. 90:2994-2998 (1993). Any physical property of the protein which changes during denaturation, e.g., the emission of light or heat, the absorption of light or heat, scattering of light, and dichroism, may be used for monitoring the structural change of the protein. Examples of such physical properties include fluorescent emission, fluorescent energy transfer, absorption of ultraviolet or visible light, changes in polarization of light, and light scattering. Fluorescence emission can be intrinsic to a protein or can be due to a fluorescence reporter molecule.

[0157] As used herein, "aggregation" refers to a physical interaction between the protein or polypeptide molecules that result in formation of multimers. The multimers may remain soluble, or precipitate out of solution.

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[0158] The multidomain protein formulation can be a solution of the multidomain protein of a suitable concentration, e.g., a concentration of 20, 30, 40, 50, 100 mg/ml or higher. The liquid multidomain protein formulation can also comprise other constituents, including but are not limited to, salts, ligands, co-factors, etc. The concentration and constituents of the liquid multidomain protein formulation are selected such that the thermal denaturation/renaturation behavior of the multidomain protein in the liquid multidomain protein formulation reflects the long term stability of the liquid multidomain protein formulation whose shelf life is to be evaluated. In one embodiment, the concentration of the multidomain protein in the liquid multidomain protein formulation being analyzed differs from the concentration of the multidomain protein in a liquid protein formulation by no more than 50%, 20%, 10%, 5% or 1%. In a specific embodiment, the concentration of the multidomain protein in the liquid multidomain protein formulation being analyzed is substantially the same as the concentration of the multidomain protein in a multidomain protein formulation.

[0159] The multidomain protein can comprise other amino acid residues or domains in addition to the aggregation-causing domain.

[0160] The concentration of a multidomain protein in a liquid formulation is at least 15 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 30 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml, at least 50 mg/ml, at least 55 mg/ml, at least 60 mg/ml, at least 65 mg/ml, at least 70 mg/ml, at least 75 mg/ml, at least 80 mg/ml, at least 85 mg/ml, at least 90 mg/ml, at least 95 mg/ml, at least 100 mg/ml, at least 105 mg/ml, at least 110 mg/ml, at least 115 mg/ml, at least 120 mg/ml, at least 125 mg/ml, at least 130 mg/ml, at least 135 mg/ml, at least 140 mg/ml, at least 150 mg/ml, at least 200 mg/ml, at least 250 mg/ml, or at least 300 mg/ml.

[0161] The formulations may also comprise other substances, e.g., histidine, glycine, saccharides (e.g., sucrose, mannose, trehalose, etc.), polyols, (e.g., Tween) and sugar alcohols (e.g., mannitol, sorbitol, etc.). The concentration of histidine which is included in the formulations ranges from 1 mM to 100 mM, or from 5 mM to 50 mM. Histidine can be in the form of L-histidine, D-histidine, or a mixture thereof, but L-histidine is generally preferred. Histidine can be also in the form of hydrates. Histidine may be used in a form of pharmaceutically acceptable salt, such as hydrochloride (e.g., monohydrochloride and dihydrochloride), hydrobromide, sulfate, acetate, etc. The purity of histidine should be at least 98%, preferably at least 99%, and most preferably at least 99.5%.

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[0162] The concentration of an amino acid to be used in a formulation can be at a concentration of less than 150 mM, less than 100 mM, less than 75 mM, less than 50 mM, less than 25 mM, less than 10 mM, less than 5.0 mM, or less than 2.0 mM. The amount of amino acid in the formulation should not cause a significant buffering effect so that protein precipitation at its isoelectric point can be avoided. An amino acid may be also used in a form of pharmaceutically acceptable salt, such as hydrochloride, hydrobromide, sulfate, acetate, etc. The purity of the amino acid should be at least 98%, or at least 99%, or at least 99.5%.

[0163] The saccharide can be sucrose at a concentration ranging from between about 1% to about 20%, preferably about 5% to about 15%, and more preferably about 8% to 10%. The polyol can be polysorbate (e.g., Tween 20) at a concentration ranging from between about 0.001% to about 1%, or about 0.01% to about 0.1%.

[0164] The pH of the formulation should not be equal to the isoelectric point of the particular multidomain protein to be used in the formulation and may range from about 5.0 to about 8.0, or about 5.5 to about 6.5, or about 6.0 to about 7.0, or about 6.5 to about 7.5, or about 7.0 to about 8.0, or about 5.8 to about 6.2, and or about 6.0.

[0165] Liquid formulations preferably exhibit stability at the temperature ranges of 38oC-42oC for at least 15 days and, in some embodiments, not more than 25 days, of 20oC-24oC for at least 1 month, at least 2 months, at least 4 months, or at least 6 months, of 2oC-8oC (in particular, at 4oC) for at least 6 months, at least 1 year, at least 1.5 years, at least 2 years, at least 3 years or at least 4 years and at -20oC for at least 1 year, at least 2 years, at least 3 years, at least 4 years, or at least 5 years, as assessed by high performance size exclusion chromatography (HPSEC). Namely, the formulations, more specifically liquid formulations, have low to undetectable levels of aggregation and/or fragmentation, as defined herein, after the storage for the defined periods as set forth above. In certain embodiments, no more than 5%, no more than 4%, no more than 3%, no more than 2%, no more than 1%, and or no more than 0.5% of the multidomain protein forms an aggregate as measured by HPSEC, after the storage for the defined periods as set forth above.

[0166] In one embodiment, a suitable solution of a multidomain protein that comprises a domain whose unfolding leads to aggregation of the multidomain protein whose shelf life is of interest is provided, wherein the solution stabilizes the domain. In another embodiment, a suitable solution of a multidomain protein comprising an engineered domain having improved long term stability is provided.

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[0167] A lyophilized formulation preferably exhibits no significant changes observed at a refrigerated temperature (2-8°C) for at least 12 months, preferably 2 years, and more preferably 3 years; or at room temperature (23-27°C) for at least 3 months, preferably 6 months, and more preferably 1 year. The criteria for stability are as follows: No more than 10%, or no more than 5%, of the protein domain is degraded as measured by HPSEC. The rehydrated solution is colorless, or clear to slightly opalescent by visual analysis. The concentration, pH and osmolality of the formulation have no more than +/- 10% change. Potency is within 70-130, preferably 80- 120 % of the control. No more than 10%, preferably 5% of clipping is observed. No more than 10%, or no more than 5% of aggregation is formed.

[0168] The methods of screening and/or evaluating the long term stability of a multidomain protein in a solution of the invention can provide an indicator of the shelf life of a plurality of formulations of different multidomain proteins, e.g., a plurality of different monoclonal antibodies. In one embodiment, a suitable solution of a multidomain protein is provided for each multidomain protein. Alternatively, the methods of screening and/or evaluating the long term stability of a multidomain protein in a solution of the invention can provide an indicator of the shelf life of a plurality of different solutions comprising the same multidomain protein. In another embodiment, a multidomain protein is formulated in a number of different solutions. In certain embodiments, each multidomain protein comprises a domain whose unfolding leads to aggregation of the corresponding multidomain protein. In other embodiments, each multidomain protein comprises a variant of a domain whose unfolding leads to aggregation of the corresponding multidomain protein. A thermal denaturation curve is measured for each of such multidomain proteins. The multidomain proteins are then ranked based on the denaturation temperatures of the multidomain proteins. The lower denaturation temperature corresponds to lower stability, and thus is indicative of a shorter shelf life.

[0169] In one embodiment, the methods of screening and/or evaluating the long term stability of a multidomain protein in a solution is used as an indicator of the shelf life of a plurality of formulations of different modified multidomain proteins. Each of the plurality of modified multidomain proteins comprises a domain in a modified form, e.g., having one or more amino acid residues in the domain substituted. A suitable solution of a multidomain protein comprising a modified domain is provided for each modified multidomain protein. A thermal denaturation curve is measured for each of such multidomain proteins. The modified

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multidomain proteins are then ranked based on the denaturation temperatures of the multidomain proteins. One or more modified monoclonal multidomain protein may be selected based on the rank order. In a specific embodiment, the multidomain protein is an antibody.

[0170] The methods of screening and/or evaluating the long term stability of a multidomain protein in a solution can also be used for identifying biochemical conditions that optimize the stability of, and hence the shelf life of, a formulation of a multidomain protein. For example, pH affects the shelf life of a protein. For a liquid formulation the composition of the solvent, e.g., water, also affect the shelf life of a protein. For example, glycerol alters the solvation properties of water to favor the native conformation of proteins. Ligands and cofactors in the solution may also affect the shelf life of a protein. Other biochemical parameters that have been shown to affect protein folding are: protein concentration, temperature, glutathione redox buffers (GSH, GSSG), the presence of detergents, and the presence of other additives, such as glycerol, arginine-HCl, polyethylene glycol (PEG), and organic solvents. Various conditions can be optimized, including pH optimum, ionic strength dependence, concentration of salts of the Hofmeister series, glycerol concentration, sucrose concentration, arginine concentration, dithiothreitol concentration, metal ion concentration, etc. Such biological conditions can be evaluated based on the thermal denaturation curve of the multidomain protein under the conditions. In one embodiment, a plurality of different conditions are ranked, and the optimal one is selected.

[0171] In one embodiment, the thermal denaturation/renaturation curve is obtained using differential scanning calorimetry (DSC). In this embodiment, a DSC thermogram of the denaturation/renaturation of the multidomain protein is obtained. Methods of using DSC to study the denaturation of proteins are well known in the art (see, e.g., Vermeer et al., 2000, Biophys. J. 78:394-404; Vermeer et al., 2000, Biophys. J. 79: 2150-2154). DSC can detect fine-tuning of interactions between the individual domains of a protein (Privalov et al., 1986, Methods Enzymol. 131:4-51).

[0172] In one embodiment, DSC measurements are performed using a Setaram Micro-DSC III (Setaram, Caluire, France). The samples are placed in the calorimeter in a 1 ml sample cell against a 1 ml reference cell containing the appropriate blank solution. The cells are stabilized for 4 h at 25°C inside the calorimeter before heating up to the final temperature at a selected heating rate. The transition temperature and enthalpy are determined using the Setaram software (Setaram, Version 1.3).

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[0173] In another embodiment, DSC measurements are performed using a VP-DSC (MicroCal, LLC). In one embodiment, a scan rate of 1.0°C/min and a temperature range of 25 –120°C are employed. A filter period of 8 seconds is used along with a 5 minute pre-scan thermostating. Multiple baselines are run with buffer in both the sample and reference cell to establish thermal equilibrium. After the baseline is subtracted from the sample thermogram, the data are concentration normalized and fitted using the deconvolution function. Melting temperatures are determined following manufacturer procedures using Origin software supplied with the system.

[0174] In another embodiment, the thermal denaturation/renaturation curve is obtained using circular dichroism (CD) spectroscopy. Changes in the secondary structure of IgG as a function of temperature and/or, e.g., pH, can be studied by CD spectroscopy (Fasman, 1996, Circular Dichroism and the Conformational Analysis of Biomolecules. Plenum Press, New York). The advantage of this technique are that the spectroscopic signal is not affected by the presence of the surrounding solution and that well-defined procedures are available to elucidate the secondary structure based on reference spectra of the different structure elements (de Jongh et al., 1994, Biochemistry. 33:14521-14528). The fractions of the secondary structural elements can be obtained from the CD spectra.

[0175] In one embodiment, the CD spectra are measured with a JASCO spectropolarimeter, model J-715 (JASCO International Co., Tokyo, Japan). A quartz cuvette of 0.1 cm light path length is used. Temperature regulation is carried out using a JASCO PTC-348WI (JASCO International) thermocouple. Temperature scans are recorded at a selected heating rate using the Peltier thermocouple with a resolution of 0.2°C and a time constant of 16 s. Wavelength scans, in the far-UV region (0.2 nm resolution) are obtained by accumulation of a plurality of scans with a suitable scan rate

[0176] The thermal denaturation/renaturation curve can also be measured by light spectrophotometry. When a protein in a solution denatures in response to heating, the molecules aggregate and the solution scatters light more strongly. Aggregation leads to changes in the optical transparency of the sample, and can be measured by monitoring the change in absorbance of visible or ultraviolet light of a defined wavelength.

[0177] In still another embodiment, fluorescence spectroscopy is used to obtain the thermal denaturation/renaturation curve. In one embodiment, intrinsic protein fluorescence, e.g., intrinsic tryptophan fluorescence, is monitored. In another embodiment, fluorescence probe molecules are monitored. Methods of performing fluorescence spectroscopy

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experiments are well known to those skilled in the art. See, for example, Bashford, C. L. et al., Spectrophotometry and Spectrofluorometry: A Practical Approach, pp. 91-114, IRL Press Ltd. (1987); Bell, J. E., Spectroscopy in Biochemistry, Vol. I, pp. 155-194, CRC Press (1981); Brand, L. et al., Ann. Rev. Biochem. 41:843 (1972).

[0178] The methods of the invention can be practiced using an array format, in which a plurality of different liquid multidomain protein preparations is evaluated simultaneously (see, U.S. Patent No. 6,232,085). The array format is particular useful for evaluating the potential shelf life of a plurality of formulations of different multidomain proteins, *e.g.*, a plurality of modified monoclonal antibodies, or the shelf life of a multidomain protein under a plurality of different biochemical conditions.

5.3. Multidomain Proteins

[0179] In one embodiment the methods of the present invention are useful for the generation of multidomain proteins with improved manufacturing characteristics (referred to herein as "multidomain proteins of the invention") that specifically bind to a target molecule. Such multidomain proteins include but are not limited to antibodies including antibody domain fusion proteins. In one embodiment, a multidomain protein of the invention specifically binds a nonpolypeptide target. In another embodiment, a multidomain protein of the invention specifically binds a polypeptide target. In another embodiment, administration of a multidomain protein of the invention to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal.

[0180] Antibody domain fusion proteins include Fc fusion proteins, which combines an Fc domain or fragment thereof, with a fusion partner, which in general can be any protein, polypeptide, peptide, including, but not limited to, the target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme, or some other protein or protein domain. Antibody domain fusion proteins also include antigen binding domain fusion proteins, which combines an antigen binding domain or fragment thereof, with a fusion partner, which in general can be any protein, polypeptide, peptide, including, but not limited to, another antigen binding domain, the target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme, a toxin or some other protein or protein domain. It will be understood that the role of the fusion partner is determined by the therapeutic goal. For example the role of the non-Fc part of an Fc fusion is generally to mediate target binding, and thus it is functionally analogous to the variable regions of an antibody while the role of the fusion partner of an antigen binding

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domain fusion protein may be to mediate binding to a second target in the case of a second antigen binding domain or to provide a cytotoxic agent in the case of a toxin.

[0181] Virtually any molecule may be targeted by and/or incorporated into a multidomain protein of the invention including, but not limited to, following list of proteins, subunits, domains, motifs, and epitopes belonging to the following list of cellular proteins: renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha- 1 - antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VII, factor VIIIC, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor (TNF) proteins such as TNF-alpha, TNF-beta, TNFbeta2, TNFc, TNFalphabeta, 4-1BBL as well as members of the TNF superfamily members such as, TNF-like weak inducer of apoptosis (TWEAK), and LIGHT, B lymphocyte stimulator (BlyS); members of the TNF receptor superfamily including TNF-RI, TNF-RII, TRAIL receptor-1, Transmembrane activator and CAML interactor (TACI) and OX40L; Fas ligand (FasL); enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors such as, for example, EGFR (ErbB-1), VGFR; interferons such as alpha interferon (α-IFN), beta interferon (β-IFN) and gamma interferon (γ-IFN); interferon alpha receptor (IFNAR) subunits 1 and/or 2 and other receptors such as, A1, Adenosine Receptor, Lymphotoxin Beta Receptor, BAFF-R, endothelin receptor; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3,-4,-5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor; platelet-derived growth factor (PDGF); fibroblast growth factor such as αFGF and βFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-1, TGF-2, TGF-3, TGF-4, or TGF-5; insulin-like growth factor-I and-II (IGF-I and IGF-II); des (1-3)-IGF-I (brain IGF-I), insulin-like growth

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factor binding proteins, keratinocyte growth factor; growth factor receptors such as, FGFR-3, IGFR; CD proteins such as CD2, CD3, CD3E, CD4, CD 8, CD11, CD11a, CD14, CD16, CD18, CD19, CD20, CD22, CD23, CD25, CD27, CD27L, CD28, CD29, CD30, CD30L, CD32, CD33 (p67 protein), CD34, CD38, CD40, CD40L, CD52, CD54, CD56, CD63, CD64, CD80 and CD147; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha,-beta, and-gamma; colony stimulating factors (CSFs), such as M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-13 and IL-15, IL-18, IL-23; EPO; superoxide dismutase; T-cell receptors alpha/beta; surface membrane proteins; decay accelerating factor; transport proteins; homing receptors; addressins; regulatory proteins; chemokine family members such as the eotaxins, the MIPs, MCP-1, RANTES; cell adhesion molecules such as selectins (L-selectin, P-selectin, E-selectin) LFA-1, LFA-3, Mac 1, p150.95, VLA-1, VLA-4, ICAM-1, ICAM-3, EpCAM and VCAM, a4/p7 integrin, and Xv/p3 integrin, integrin alpha subunits such as CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, alpha7, alpha8, alpha9, alphaD, CD11a, CD11b, CD51, CD11c, CD41, alphaIIb, alphaIELb; integrin beta subunits such as, CD29, CD 18, CD61, CD104, beta5, beta6, beta7 and beta8; Integrin subunit combinations including but not limited to, αVβ3, αVβ5 and α4β7; cellular ligands such as, TNF-related apoptosis-inducing ligand (TRAIL), A proliferation-inducing ligand (APRIL), B Cell Activating Factor (BAFF), a member of an apoptosis pathway; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C; an Eph receptor such as EphA2, EphA4, EphB2, etc.; immune system markers, receptors and ligands such as CTLA-4, T cell receptor, B7-1, B7-2, IgE, Human Leukocyte Antigen (HLA) such as HLA-DR, CBL; complement proteins such as complement receptor CR1, C1Rq and other complement factors such as C3, and C5; blood factors including tissue factor, factor VII; a glycoprotein receptor such as GpIbα, GPIIb/IIIa and CD200; and fragments of any of the above-listed polypeptides.

[0182] Also contemplated are multidomain proteins of the invention that specifically bind cancer antigens including, but not limited to, ALK receptor (pleiotrophin receptor), pleiotrophin; KS 1/4 pan-carcinoma antigen; ovarian carcinoma antigen (CA125); prostatic acid phosphate; prostate specific antigen (PSA); melanoma-associated antigen p97; melanoma antigen gp75; high molecular weight melanoma antigen (HMW-MAA); prostate specific membrane antigen; carcinoembryonic antigen (CEA); carcinoembryonic antigen-related cell adhesion molecule (CEACAM1); cytokeratin tumor-associated antigen; human milk fat globule (HMFG) antigen; tumor-associated antigen expressing Lewis Y related

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carbohydrate; colorectal tumor-associated antigens such as: CEA, tumor-associated glycoprotein-72 (TAG-72), CO17-1A, GICA 19-9, CTA-1 and LEA; Burkitt's lymphoma antigen-38.13; CD19; human B-lymphoma antigen-CD20; CD33; melanoma specific antigens such as ganglioside GD2, ganglioside GD3, ganglioside GM2 and ganglioside GM3; tumor-specific transplantation type cell-surface antigen (TSTA); virally-induced tumor antigens including T-antigen, DNA tumor viruses and Envelope antigens of RNA tumor viruses; oncofetal antigen-alpha-fetoprotein such as CEA of colon, 5T4 oncofetal trophoblast glycoprotein and bladder tumor oncofetal antigen; differentiation antigen such as human lung carcinoma antigens L6 and L20; antigens of fibrosarcoma; human leukemia T cell antigen-Gp37; neoglycoprotein; sphingolipids; breast cancer antigens such as EGFR (Epidermal growth factor receptor); NY-BR-16; NY-BR-16 and HER2 antigen (p185HER2); Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), polymorphic epithelial mucin (PEM) antigen; epithelial membrane antigen (EMA); Melanoma-associated antigen MUC18; MUC1; malignant human lymphocyte antigen-APO-1; differentiation antigen such as I antigen found in fetal erythrocytes; primary endoderm I antigen found in adult erythrocytes; preimplantation embryos; I(Ma) found in gastric adenocarcinomas; M18, M39 found in breast epithelium; SSEA-1 found in myeloid cells; VEP8; VEP9; Myl; VIM-D5; D156-22 found in colorectal cancer; TRA-1-85 (blood group H); SCP-1 found in testis and ovarian cancer; C14 found in colonic adenocarcinoma; F3 found in lung adenocarcinoma; AH6 found in gastric cancer; Y hapten; Ley found in embryonal carcinoma cells; TL5 (blood group A); EGF receptor found in A431 cells; E1 series (blood group B) found in pancreatic cancer; FC10.2 found in embryonal carcinoma cells; gastric adenocarcinoma antigen; CO-514 (blood group Lea) found in Adenocarcinoma; NS-10 found in adenocarcinomas; CO-43 (blood group Leb); G49 found in EGF receptor of A431 cells; MH2 (blood group ALeb/Ley) found in colonic adenocarcinoma; 19.9 found in colon cancer; gastric cancer mucins; T5A7 found in myeloid cells; R24 found in melanoma; 4.2, GD3, D1.1, OFA-1, GM2, OFA-2, GD2, and M1:22:25:8 found in embryonal carcinoma cells and SSEA-3 and SSEA-4 found in 4 to 8-cell stage embryos; Cutaneous Tcell Lymphoma antigen; MART-1 antigen; Sialy Tn (STn) antigen; Anaplastic lymphoma kinase (ALK) found in large cell lymphoma; Colon cancer antigen NY-CO-45; Lung cancer antigen NY-LU-12 variant A; Adenocarcinoma antigen ART1; Paraneoplastic associated brain-testis-cancer antigen (onconeuronal antigen MA2; paraneoplastic neuronal antigen); Neuro-oncological ventral antigen 2 (NOVA2); Hepatocellular carcinoma antigen gene 520; TUMOR-ASSOCIATED ANTIGEN CO-029; Tumor-associated antigens MAGE-C1 (cancer/testis antigen CT7), MAGE-B1 (MAGE-XP

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antigen), MAGE-B2 (DAM6), MAGE-2, MAGE-4a, MAGE-4b and MAGE-X2; Cancer-Testis Antigen (NY-EOS-1); placental alkaline phosphatase (PLAP) and testicular PLAP-like alkaline phosphatase, transferrin receptor; Heparanase I; EphA2 associated with numerous cancers and fragments of any of the above-listed polypeptides.

[0183] Other exemplary polypeptide targets include but not limited to the following list of proteins, subunits, domains, motifs, and epitopes belonging to the following list of microbial proteins: human cytomegalovirus (HCMV) proteins such as, envelope glycoprotein, gB, internal matrix proteins of the virus, pp65 and pp150, immediate early (IE) proteins; human immunodeficiency virus (HIV) proteins such as, Gag, Pol, Vif and Nef (Vogt et al., 1995, Vaccine 13: 202-208); HIV antigens gp120 and gp160 (Achour et al., 1995, Cell. Mol. Biol. 41: 395-400; Hone et al., 1994, Dev. Biol. Stand. 82: 159-162); gp41 epitope of human immunodeficiency virus (Eckhart et al., 1996, J. Gen. Virol. 77: 2001-2008); hepatitis C virus (HCV) proteins such as, nucleocapsid protein in a secreted or a nonsecreted form, core protein (pC); E1 (pE1), E2 (pE2) (Saito et al., 1997, Gastroenterology 112: 1321-1330), NS3, NS4a, NS4b and NS5 (Chen et al., 1992, Virology 188:102-113); severe acute respiratory syndrome (SARS) corona virus proteins include but are not limited to, the S (spike) glycoprotein, small envelope protein E (the E protein), the membrane glycoprotein M (the M protein), the hemagglutinin esterase protein (the HE protein), and the nucleocapsid protein (the N-protein) See, e.g., Marra et al., "The Genome Sequence of the SARS-Associated Coronavirus," Science Express, May 2003); Mycobacterium tuberculosis proteins such as the 30-35 kDa (a.k.a. antigen 85, alpha-antigen) that is normally a lipoglycoprotein on the cell surface, a 65-kDa heat shock protein, and a 36-kDa proline-rich antigen (Tascon et al. (1996) Nat. Med. 2: 888-92), Ag85A, Ag85b (Huygen et al., 1996, Nat. Med. 2: 893-898), 65-kDa heat shock protein, hsp65 (Tascon et al., 1996, Nat. Med. 2: 888-892), MPB/MPT51 (Miki et al., 2004, Infect. Immun. 72:2014-21), MTSP11, MTSP17 (Lim et al., 2004, FEMS Microbiol. Lett. 232:51-9 and supra); Herpes simplex virus (HSV) proteins such as gD glycoprotein, gB glycoprotein; proteins from intracellular parasites such as Leishmania include LPG, gp63 (Xu and Liew, 1994, Vaccine 12: 1534- 1536; Xu and Liew, 1995, Immunology 84: 173-176), P-2 (Nylen et al., 2004, Scand. J. Immunol. 59:294-304), P-4 (Kar et al. 2000, J Biol. Chem. 275:37789-97), LACK (Kelly et al., 2003, J Exp. Med. 198:1689-98); microbial toxin proteins such as Clostridium perfringens toxin; in addition, exemplary antigen peptides of human respiratory syncytial virus (hRSV), human

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metapneumovirus (HMPV) and Parainfluenza virus (PIV) are detailed in: Young et al., in Patent publication WO04010935A2.

[0184] One skilled in the art will appreciate that the aforementioned lists of targets refers not only to specific proteins and biomolecules, but the biochemical pathway or pathways that comprise them. For example, reference to CTLA-4 as a target antigen implies that the ligands and receptors that make up the T cell co-stimulatory pathway, including CTLA-4, B7-1, B7-2, CD28, and any other undiscovered ligands or receptors that bind these proteins, are also targets. Thus target, as used herein, refers not only to a specific biomolecule, but the set of proteins that interact with the target and the members of the biochemical pathway to which the target belongs. One skilled in the art will further appreciate that any of the aforementioned target antigens, the ligands or receptors that bind them, or other members of their corresponding biochemical pathway, may be operably linked to the multidomain proteins or a portion thereof of the present invention in order to generate a fusion protein. Thus for example, a multidomain fusion that targets EGFR could be constructed by operably linking a multidomain protein or portion thereof to EGF, TGFα, or any other ligand, discovered or undiscovered, that binds EGFR. Accordingly, a multidomain protein or portion thereof of the present invention could be operably linked to EGFR in order to generate an fusion that binds EGF, TGFα, or any other ligand, discovered or undiscovered, that binds EGFR. Thus virtually any polypeptide, whether a ligand, receptor, or some other protein or protein domain, including but not limited to the aforementioned targets and the proteins that compose their corresponding biochemical pathways, may be operably linked to the multidomain proteins of the present invention or a portion thereof to develop a fusion.

[0185] In one embodiment the multidomain protein of the invention is an antibody or an antibody domain fusion protein. Numerous methods useful for the generation of antibodies or antibody domain fusion proteins are well known in the art. Several nonlimiting examples are described below in section 5.4. In a specific embodiment the methods of the present invention are utilized to generate and/or screen antibody or antibody domain fusion proteins which have preferred/improved manufacturing characteristics.

[0186] A number of specific multidomain proteins, namely antibodies and antibody domain fusion proteins (e.g., Fc fusions) that are approved for use, in clinical trials, or in development may benefit from the methods of the present invention. Said antibodies and antibody domain fusion proteins (e.g., Fc fusions) are herein referred to as "clinical products and candidates". Thus in specific embodiments, the methods of the present invention may

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find use in a range of clinical products and candidates. For example the engineering and screening methods of the present invention may find use developing an antibody with improved manufacturing characteristics that has binding and functional characteristics substantially similar to rituximab (Rituxan®, IDEC/Genentech/Roche) (see for example U.S. Pat. No. 5,736,137), a chimeric anti-CD20 IgG1 antibody approved to treat Non-Hodgkin's lymphoma; HuMax-CD20 (Genmab), an anti-CD20 (see for example PCT WO 04/035607); an anti-CD20 antibody described in U.S. Pat. No. 5,500,362; AME-I33 (Applied Molecular Evolution) humanized and optimized anti-CD20 Mab; hA20 (Immunomedics, Inc.) a humanized anti-CD20 Mab; HumaLYM™ (Intracel) a fully human anti-CD20 Mab; trastuzumab (Herceptin®, Genentech) a humanized anti-Her2/neu antibody approved to treat breast cancer (see for example U.S. Pat. No. 5,677,171); pertuzumab (rhuMab-2C4, Omnitarg™, Genentech); an anti-Her2 antibody described in U.S. Pat. No. 4,753,894; cetuximab (Erbitux®, Imclone) (U.S. Pat. No. 4,943,533; PCT WO 96/40210), a chimeric anti-EGFR antibody in clinical trials for a variety of cancers; ABX-EGF (Abgenix/Immunex/Amgen) described in U.S. Pat. No. 6,235,883; HuMax-EGFr (Genmab) described in U.S. patent application No. 10/172,317; 425, EMD55900, EMD62000, and EMD72000 (Merck KGaA) (U.S. Pat. No. 5,558,864); ICR62 (Institute of Cancer Research) (PCT WO 95/20045); TheraCIM hR3 (YM Biosciences, Canada and Centro de Immunologia Molecular, Cuba) (U.S. Pat. Nos. 5,891,996; 6,506,883); mAb-806 (Ludwig Institute for Cancer Research, Memorial Sloan-Kettering) (Jungbluth et al. 2003, Proc Natl Acad Sci USA. 100(2):639-44); KSB-102 (KS Biomedix); MR1-1 (IVAX, National Cancer Institute) (PCT WO 01/62931); and SC100 (Scancell) (PCT WO 01/88138); alemtuzumab (Campath®, Genzyme), a humanized monoclonal anti CD52 IgG1 antibody currently approved for treatment of B-cell chronic lymphocytic leukemia; muromonab-CD3 (Orthoclone OKT3®, Ortho Biotech/Johnson & Johnson), an anti-CD3 antibody; OrthoClone OKT4A (Ortho Biotech), a humanized anti-CD4 IgG antibody; ibritumomab tiuxetan (Zevalin®, IDEC/Schering AG), a radiolabeled anti-CD20 antibody; gemtuzumab ozogamicin (Mylotarg®, Celltech/Wyeth), an anti-CD33 (p67 protein) antibody; alefacept (Amevive®, Biogen), an anti-LFA-3 Fc fusion; abciximab (ReoPro®, Centocor/Lilly), a anti-glycoprotein IIb/IIIa receptor on the platelets for the prevention of clot formation; basiliximab (Simulect®, Novartis) an anti-CD25 antibody; infliximab (Remicade®, Centocor), an anti-TNFalpha antibody; adalimumab (Humira®, Abbott), an anti-TNFalpha antibody; Humicade™ (CellTech), an anti-TNFalpha antibody; etanercept (Enbrel®, Immunex/Amgen), an anti-TNFalpha Fc fusion; ABX-CBL (Abgenix), an anti-CD147 antibody; ABX-IL8 (Abgenix),

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an anti-lL8 antibody; ABX-MA1 (Abgenix), an anti-MUC18 antibody; Pemtumomab (R1549, 90Y-muHMFG1, Antisoma), an anti-MUC1 antibody; Therex (R1550, Antisoma), an anti-MUC1 antibody; AngioMab (AS1405, Antisoma), HuBC-1 and Thioplatin (AS1407) being developed by Antisoma; natalizumab (Antegren®, Biogen), an anti-alpha-4-beta-1 (VLA-4) and alpha-4-beta-7 antibody; ANTOVA™ (Biogen). a humanized anti-CD40L IgG antibody; VLA-1 mAb (Biogen), an anti-VLA-1 integrin antibody; LTBR mAb (Biogen), an anti-lymphotoxin beta receptor (LTBR) antibody; CAT-152 (Cambridge Antibody Technology), an anti-TGFβ2 antibody; J695 (Cambridge Antibody Technology/Abbott), an anti-IL-12 antibody; CAT-192 (Cambridge Antibody Technology/Genzyme); an anti-TGFβ1 antibody; CAT-213 (Cambridge Antibody Technology), an anti-Eotaxin1 antibody; LymphoStat-B™ an anti-Blys antibody and TRAIL-R1mAb, an anti-TRAIL-R1 antibody both being developed by Cambridge Antibody Technology and Human Genome Sciences, Inc.; bevacizumab (Avastin™, rhuMAb-VEGF, Genentech) an anti-VEGF antibody; an anti-HER receptor family antibody (Genentech); Anti-Tissue Factor antibody (Genentech); Omalizumab (Xolair™, Genentech) an anti-IgE antibody; Efalizumab (Raptiva™, Genentech/Xoma), an anti-CD11a antibody; MLN-02 Antibody (formerly LDP-02, Genentech /Millenium Pharmaceuticals), a humanized anti-α4β7 antibody; HuMax CD4 (Genmab), an anti-CD4 antibody being; HuMax-IL 15 (Genmab and Amgen), an anti-IL15 antibody; HuMax-Inflam (Genmab/Medarex),; HuMax-Cancer (Genmab/Medarex/Oxford GcoSciences), an anti-Heparanase I antibody; HuMax-Lymphoma (Genmab/Amgen); HuMax-TAC (Genmab); IDEC-131 (IDEC Pharmaceuticals), an anti-CD40L antibody; Clenoliximab (IDEC-151, IDEC Pharmaceuticals), an anti-CD4 antibody; IDEC-114 (IDEC Pharmaceuticals), an anti-CD80 antibody; IDEC-152 (IDEC Pharmaceuticals), an anti-CD23; anti-macrophage migration factor (MIF) antibodies being developed by IDEC Pharmaceuticals; BEC2 (Imclone), an anti-idiotypic antibody; IMC-1C11 (Imclone), an anti-KDR antibody; DC101 (Imclone), an anti-flk-1 antibody; anti-VE cadherin antibodies being developed by Imclone; labetuzumab (CEA-Cide™, Immunomedics), an anti-carcinoembryonic antigen (CEA) antibody; Epratuzumab (LymphoCide™, Immunomedics), an anti-CD22 antibody; AFP-Cide (Immunomedics); MyelomaCide (Immunomedics); LkoCide (Immunomedics); ProstaCide (Immunomedics); MDX-010 (Medarex), an anti-CTLA4 antibody; MDX-060 (Medarex), an anti-CD30 antibody; MDX-070 (Medarex); MDX-018 (Medarex); MDX-CD4 (Medarex/Eisai/Genmab), a human anti-CD4 IgG antibody; Osidem™ (IDM-1, Medarex/Immuno-Designed Molecules), an anti-Her2 antibody; HuMax™-CD4 (Medarex/Genmab), an anti-CD4 antibody; HuMax-IL15

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(Medarex/Genmab); CNTO 148 (Medarex/Centocor/J&J), an anti-TNFα antibody; CNTO 1275 (Centocor/J&J), an anti-cytokine antibody; CNTO 95 (Centocor/J&J), a human Integrin αv antibody (PCT publication WO 02/12501); MOR101 and MOR102 (MorphoSys), anti-intercellular adhesion molecule-1 (ICAM-1) (CD54) antibodies; MOR201 (MorphoSys), an anti-fibroblast growth factor receptor 3 (FGFR-3) antibody; visilizumab (Nuvion®, Protein Design Labs), an anti-CD3 antibody; HuZAF™ (Protein Design Labs), an anti-gamma interferon antibody; Anti-α5β1 Integrin (Protein Design Labs); anti-IL-12 (Protein Design Labs); ING-1 (Xoma), an anti-Ep-CAM antibody; MLN01 (Xoma), an anti-Beta2 integrin antibody; ZENAPAX® (daclizumab, Roche Pharmaceuticals) an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection; CDP860 (Celltech, UK), a humanized, PEGylated anti-CD18 F(ab')2; PRO542 (Progenics/Genzyme Transgenics), an anti-HIV gp120 antibody fused with CD4; C14 (ICOS Pharm), an anti-CD14 antibody; OVAREX™ (Altarex), a murine anti-CA 125 antibody; PANOREX™ (Glaxo Wellcome/Centocor), a murine anti-17-IA cell surface antigen IgG2a antibody; VITAXIN™ (MedImmune, PCT publication No. WO 2003/075957), a humanized anti-αVβ3 integrin antibody; siplizumab (MEDI-507, MedImmune, WO 99/03502), a humanized form of the murine monoclonal anti-CD2 antibody, BTI-322; palivizumab (Synagis®, MedImmune), a humanized neutralizing anti-RSV antibody; MEDI-524 (Numax, MedImmune), an affinity optimized humanized anti RSV antibody; Zamyl (Smart M195, Protein Design Lab/Kanebo), a humanized anti-CD33 IgG antibody; Remitogen (Smart 1D10, Protein Design Lab/Kanebo) which is a humanized anti-HLA antibody; ONCOLYM™ (Lym-1, Techniclone) is a radiolabelled murine anti-HLA DR antibody; Efalizumab (Genetech/Xoma), a humanized monoclonal anti-CD11a antibody; ICM3 (ICOS Pharm), a humanized anti-ICAM3 antibody; IDEC-114 (IDEC Pharm/Mitsubishi), a primatized anti-CD80 antibody; eculizumab (5G1.1, Alexion Pharm) a humanized anti-complement factor 5 (C5) antibody; pexelizumab (5G1.1-SC, Alexion Pharm) a fully humanized single chain monoclonal antibody; LDP-01 (Millennium/Xoma), a humanized anti-β2-integrin IgG antibody. In specific embodiments, the multidomain proteins of the invention are not Numax® or an antigen-binding fragment thereof (*e.g.*, a Fab fragment of Numax®).

5.4. Generation Of Antibodies

[0187] In one embodiment, the multidomain proteins of the invention are antibodies that specifically bind to a target molecule. Nonlimiting examples of target molecules are

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described above (see section 5.3). Antibodies that specifically bind any target (also referred to herein as "antigen") can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

[0188] Polyclonal antibodies specific for an antigen can be produced by various procedures well-known in the art. For example, a human antigen can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the human antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

[0189] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) . The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0190] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. Briefly, mice can be immunized with a non-murine antigen and once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding the antigen. Ascites fluid,

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which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

[0191] Monoclonal antibodies can be generated by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with a non-murine antigen with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind to the antigen.

[0192] Antibody fragments which recognize specific particular epitopes (referred to herein as "antigen binding domain(s)") may be generated by any technique known to those of skill in the art. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain. Further, the antibodies of the present invention can also be generated using various phage display methods known in the art.

[0193] In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In particular, DNA sequences encoding VH and VL domains are amplified from animal cDNA libraries (e.g., human or murine cDNA libraries of affected tissues). The DNA encoding the VH and VL domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector. The vector is electroporated in E. coli and the E. coli is infected with helper phage. Phage used in these methods are typically filamentous phage including fd and M13 and the VH and VL domains are usually recombinantly fused to either the phage gene III or gene VIII. Phage expressing an antigen binding domain that binds to a particular antigen can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., 1995, J. Immunol. Methods 182:41-50; Ames et al., 1995, J. Immunol. Methods 184:177-186; Kettleborough et al., 1994, Eur. J. Immunol. 24:952-958; Persic et al., 1997, Gene 187:9-18; Burton et al., 1994, Advances in Immunology 57:191-280; International application No. PCT/GB91/O1 134; International publication Nos. WO 90/02809, WO 91/10737, WO 92/01047, WO 92/18619, WO 93/11236, WO 95/15982, WO 95/20401, and WO97/13844; and U.S. Patent Nos. 5,698,426, 5,223,409, 5,403,484, 5,580,717, 5,427,908,

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5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727, 5,733,743 and 5,969,108.

[0194] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described below. Techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication No. WO 92/22324; Mullinax et al., 1992, BioTechniques 12(6):864-869; Sawai et al., 1995, AJRI 34:26-34; and Better et al., 1988, Science 240:1041-1043.

[0195] To generate whole antibodies, PCR primers including VH or VL nucleotide sequences, a restriction site, and a flanking sequence to protect the restriction site can be used to amplify the VH or VL sequences in scFv clones. Utilizing cloning techniques known to those of skill in the art, the PCR amplified VH domains can be cloned into vectors expressing a VH constant region, e.g., the human gamma 4 constant region, and the PCR amplified VL domains can be cloned into vectors expressing a VL constant region, e.g., human kappa or lamba constant regions. Preferably, the vectors for expressing the VH or VL domains comprise a promoter, a secretion signal, a cloning site for the variable domain, constant domains, and a selection marker such as neomycin. The VH and VL domains may also cloned into one vector expressing the necessary constant regions. The heavy chain conversion vectors and light chain conversion vectors are then co-transfected into cell lines to generate stable or transient cell lines that express full-length antibodies, e.g., IgG, using techniques known to those of skill in the art.

[0196] Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al.,1983, Nature, 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., 1991, EMBO J., 10:3655-3659. A more directed approach is the generation of a Di-diabody a tetravalent

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bispecific antibody. Methods for producing a Di-diabody are known in the art (see e.g., Lu et al., 2003, J Immunol Methods 279:219-32; Marvin et al., 2005, Acta Pharmacolical Sinica 26:649).

[0197] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when, the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0198] In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., 1986, Methods in Enzymology, 121:210. According to another approach described in WO96/27011, a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine

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or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0199] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[0200] For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use humanized antibodies or chimeric antibodies. Completely human antibodies and humanized antibodies are particularly desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also U.S. Patent Nos. 4,444,887 and 4,716,111; and International publication Nos. WO 98/46645, WO 98/50433, WO 98/24893, WO98/16654, WO 96/34096, WO 96/33735, and WO 91/10741.

[0201] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional

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hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, Int. Rev. Immunol. 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Patent Nos. 5,413,923, 5,625,126, 5,633,425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939,598. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0202] A chimeric antibody is a molecule in which different portions of the antibody are derived from different immunoglobulin molecules. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, 1985, Science 229:1202; Oi et al., 1986, BioTechniques 4:214; Gillies et al., 1989, J. Immunol. Methods 125:191-202; and U.S. Patent Nos. 5,807,715, 4,816,567, 4,8 16397, and 6,311,415.

[0203] A humanized antibody is an antibody or its variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a framework region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', F(ab').sub.2, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. Preferably, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and lgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG1. Where such

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cytotoxic activity is not desirable, the constant domain may be of the IgG2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art. The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, e.g., the donor CDR or the consensus framework may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or framework residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental framework and CDR sequences, more often 90%, and most preferably greater than 95%. A humanized antibody can be produced using variety of techniques known in the art, including but not limited to, CDR-grafting (see e.g., European Patent No. EP 239,400; International Publication No. WO 91/09967; and U.S. Patent Nos. 5,225,539, 5,530,101, and 5,585,089), veneering or resurfacing (see e.g., European Patent Nos. EP 592,106 and EP 519,596; Padlan, 1991, Molecular Immunology 28(4/5):489-498; Studnicka et al., 1994, Protein Engineering 7(6):805-814; and Roguska et al., 1994, PNAS 91:969-973), chain shuffling (see e.g., U.S. Patent No. 5,565,332), and techniques disclosed in, e.g., U.S. Pat. No. 6,407,213, U.S. Pat. No. 5,766,886, International Publication No. WO 9317105, Tan et al., J. Immunol. 169:1119-25 (2002), Caldas et al., Protein Eng. 13(5):353-60 (2000), Morea et al., Methods 20(3):267-79 (2000), Baca et al., J. Biol. Chem. 272(16):10678-84 (1997), Roguska et al., Protein Eng. 9(10):895-904 (1996), Couto et al., Cancer Res. 55 (23 Supp):5973s - 5977s (1995), Couto et al., Cancer Res. 55(8):1717-22 (1995), Sandhu JS, Gene 150(2):409-10 (1994), and Pedersen et al., J. Mol. Biol. 235(3):959-73 (1994). Often, framework residues in the framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; and Riechmann et al., 1988, Nature 332:323.)

[0204] Further, the antibodies of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" a receptor using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1989, FASEB J. 7(5): 437-444; and Nissinoff,

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1991, J. Immunol. 147(8): 2429-2438). For example, antibodies of the invention which bind to and competitively inhibit the binding of an receptor (as determined by assays well known in the art and disclosed infra) to its ligands can be used to generate anti-idiotypes that "mimic" the ligand and, as a consequence, bind to and neutralize the receptor and/or its ligands. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize a ligand and/or its receptor. The invention provides methods employing the use of polynucleotides comprising a nucleotide sequence encoding an antibody of the invention or a fragment thereof.

[0205] The invention provides polynucleotides comprising a nucleotide sequence encoding an antibody or fragment thereof that specifically binds to an antigen and having one or more amino acid substitutes for improved therapeutic and/or formulation and/or manufacturing characteristics. The invention also encompasses polynucleotides that hybridize under high stringency, intermediate or lower stringency hybridization conditions to polynucleotides that encode an antibody of the invention.

[0206] The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. The nucleotide sequence of antibodies specific for a desired antigen can be obtained, e.g., from the literature or a database such as GenBank. Nucleotide or alternatively, if the amino acid sequence of an antibody or fragment thereof that specifically binds to an antigen is known, the nucleotide sequences encoding the antibody or a fragment thereof (e.g., a CDR) can be determined using methods well known in the art, i.e., nucleotide codons known to encode particular amino acids are assembled in such a way to generate a nucleic acid that encodes the antibody. Such a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., 1994, BioTechniques 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

[0207] Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as

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hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

[0208] Once the nucleotide sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Current Protocols in Molecular Biology, F.M. Ausubel et al., ed., John Wiley & Sons (Chichester, England, 1998); Molecular Cloning: A Laboratory Manual, 3rd Edition, J. Sambrook et al., ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY, 2001); Antibodies: A Laboratory Manual, E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY, 1988); and Using Antibodies: A Laboratory Manual, E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, 1999)), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or inscrtions.

[0209] In a specific embodiment, one or more of the CDRs is inserted within framework regions using routine recombinant DNA techniques. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., 1998, J. Mol. Biol. 278: 457-479 for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds to a particular antigen. Preferably, as discussed supra, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

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5.5. Generation Of Antibody Domain Fusion Proteins Conjugates And Derivatives

[0210] As described above multidomain proteins include but are not limited to antibodies which also encompasses antibody-like and antibody-domain fusion proteins. An antibody-like molecule is any molecule that has been generated with a desired binding property, see, e.g., PCT Publication Nos. WO 04/044011; WO 04/058821; WO 04/003019 and WO 03/002609. Antibody-domain fusion proteins may incorporate one or more antibody domains or fragments thereof, such as the Fc domain or the variable domain with a fusion partner which in general can be an protein, including, but not limited to, a ligand, an enzyme, the ligand portion of a receptor, an adhesion protein, or some other protein or domain. For example, the heterologous polypeptides may be fused or conjugated to a Fab fragment, Fd fragment, Fv fragment, F(ab)2 fragment, a VH domain, a VL domain, a VH CDR, a VL CDR, or fragment thereof. A large number of antibody-domain molecules are known in the art including, but not limited to, diabodies (dsFv)2 (Bera et al., 1998, J. Mol. Biol. 281:475-83); minibodies (homodimers of scFv-CH3 fusion proteins)(Pessi et al., 1993, Nature 362:367-9), tetravalent di-diabody (Lu et al., 2003 J. Immunol. Methods 279:219-32), tetravalent bi-specific antibodies called Bs(scFv)4-IgG (Zuo et al., 2000, Protein Eng. 13:361-367) and Fc domain fusions (See, e.g., Chamow et al., 1996, Trends Biotechnol 14:52-60; Ashkenazi et al., 1997, Curr Opin Immunol 9:195-200; Heidaran et al., 1995, FASEB J. 9:140-5.).

[0211] In one embodiment, a multidomain comprises an antibody domain that binds to a molecule (i.e., target antigen) including, but not limited to, those disclosed herein (see Section 5.3) fused to a fusion partner. Fusion partners include, but are not limited to, peptides, polypeptides, proteins, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. In one embodiment, a fusion partner is a polypeptide comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 contiguous amino acid residues, and is heterologous to the amino acid sequence of the antibody domain. In certain embodiments, said fusion partner is a bioactive molecule. The nucleotide sequences encoding a bioactive molecule may be obtained from any information available to those of skill in the art (i.e., from Genbank, the literature, or by routine cloning).

[0212] The fusion does not necessarily need to be direct, but may occur through linker sequences. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res 4:2483; Peterson et al., 1999, Bioconjug Chem 10:553;

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Zimmerman et al., 1999, Nucl Med Biol 26:943; Garnett, 2002, Adv Drug Deliv Rev 53:171. For example, antibody domains may be used to target heterologous polypeptides to particular cell types, either in vitro or in vivo, by fusing or conjugating the heterologous protein to antibodies domains specific for particular cell surface receptors. Antibody domains fused or conjugated to heterologous polypeptides may also be used in in vitro immunoassays and purification methods using methods known in the art. See e.g., International publication No. WO 93/21232; European Patent No. EP 439,095; Naramura et al., 1994, Immunol. Lett. 39:91-99; U.S. Patent No. 5,474,981; Gillies et al., 1992, PNAS 89:1428-1432; and Fell et al., 1991, J. Immunol. 146:2446-2452.

[0213] Methods for fusing or conjugating polypeptides to antibody portions are well known in the art. See, e.g., U.S. Patent Nos. 5,336,603, 5,783,181, 5,622,929, 5,359,046, 5,349,053, 5,447,851, 5,723,125, 5,908,626, 5,844,095, and 5,112,946; European Patent Nos. EP 307,434 and EP 367,166 and EP 394,827; PCT Publication Nos. WO 96/04388, WO 91/06570 WO 96/22024, WO 97/34631, and WO 99/04813; Ashkenazi et al., 1991, Proc. Natl. Acad. Sci. USA 88: 10535-10539; Traunecker et al., 1988, Nature, 331:84-86; Zheng et al., 1995, J. Immunol. 154:5590-5600; and Vil et al., 1992, Proc. Natl. Acad. Sci. USA 89:11337- 11341.

[0214] Multidomain proteins (e.g., an antibody domain fusion protein) can be produced by standard recombinant DNA techniques or by protein synthetic techniques, e.g., by use of a peptide synthesizer. For example, a nucleic acid molecule encoding a multidomain protein (e.g., an antibody domain fusion protein) can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). The nucleotide sequence coding for a multidomain protein (e.g., an antibody domain fusion protein) can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors; or

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bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

[0215] Additional fusion proteins, e.g., of antibody domains that specifically bind an antigen (e.g., supra), may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to alter the activities of antibodies of the invention or fragments thereof (e.g., antibodies or fragments thereof with higher affinities and lower dissociation rates). See, generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., 1997, Curr. Opinion Biotechnol. 8:724-33; Harayama, 1998, Trends Biotechnol. 16(2): 76-82; Hansson, et al., 1999, J. Mol. Biol. 287:265-76; and Lorenzo and Blasco, 1998, Biotechniques 24(2): 308- 313. Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding an antibody or antibody fragment, which portions specifically bind to an Antigen may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

[0216] Moreover, antibodies or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification. In certain embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, Cell 37:767) and the "flag" tag.

[0217] Antibodies and antibody domain fusion proteins include derivatives that are modified (i.e., by the covalent attachment of any type of molecule to an antibody or antibody domain fusion protein) such derivatives are collectively referred to herein as "antibody derivatives". For example, but not by way of limitation, the antibody derivatives include antibodies and antibody domain fusion proteins that have been modified, e.g., by

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glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0218] Antibodies or antibody domain fusion proteins with increased in vivo half-lives can be generated by attaching to said antibodies or antibody domain fusion proteins, polymer molecules such as high molecular weight polyethyleneglycol (PEG). PEG can be attached to said antibodies or antibody domain fusion proteins with or without a multifunctional linker either through site-specific conjugation of the PEG to the N– or C-terminus of said antibodies or antibody fragments or via epsilon-amino groups present on lysine residues. Linear or branched polymer derivatization that results in minimal loss of biological activity will be used. The degree of conjugation will be closely monitored by SDS-PAGE and mass spectrometry to ensure proper conjugation of PEG molecules to the antibodies. Unreacted PEG can be separated from antibody-PEG conjugates by, e.g., size exclusion or ion-exchange chromatography.

[0219] Further, antibodies or antibody domain fusion proteins can be conjugated to albumin in order to make them more stable in vivo or have a longer half life in vivo. The techniques are well known in the art, see e.g., International Publication Nos. WO 93/15199, WO 93/15200, and WO 01/77137; and European Patent No. EP 413, 622. The present invention encompasses the use of antibodies or antibody domain fusion proteins conjugated or fused to one or more moieties, including but not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules.

[0220] In certain embodiments, antibody domains are conjugated to a diagnostic or detectable agent. Such multidomain proteins can be useful for monitoring or prognosing the development or progression of a cancer as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can be accomplished by coupling the antibody to detectable substances including, but not limited to various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidin/biotin and avidin/biotin; fluorescent materials, such as but not limited to,

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umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not limited to iodine (131I, 125I, 123I, 121I,), carbon (14C), sulfur (35S), tritium (3H), indium (115In, 113In, 112In, 111In,), and technetium (99Tc), thallium (201Ti), gallium (68Ga, 67Ga), palladium (103Pd), molybdenum (99Mo), xenon (133Xe), fluorine (18F), 153Sm, 177Lu, 159Gd, 149Pm, 140La, 175Yb, 166Ho, 90Y, 47Sc, 186Re, 188Re,142 Pr, 105Rh, 97Ru, 68Ge, 57Co, 65Zn, 85Sr, 32P, 153Gd, 169Yb, 51Cr, 54Mn, 75Se, 113Sn, and 117Tin; positron emitting metals using various positron emission tomographies, noradioactive paramagnetic metal ions, and molecules that are radiolabelled or conjugated to specific radioisotopes.

[0221] An antibody or an antibody domain may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include ribonuclease, monomethylauristatin E and F, paclitaxel, 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, puromycin, epirubicin, and cyclophosphamide and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine 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). A more extensive list of therapeutic moieties can be found in PCT publications WO 03/075957.

[0222] Further, an antibody or antibody domain may be conjugated to a therapeutic agent or drug moiety that modifies a given biological response. Therapeutic agents or drug moieties are 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, a toxin such as abrin, ricin A, Onconase (or

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another cytotoxic RNase), pseudomonas exotoxin, cholera toxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-α, TNF-β, AIM I (see, International Publication No. WO 97/33899), AIM II (see, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., 1994, J. Immunol., 6:1567), and VEGI (see, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, a biological response modifier such as, for example, a lymphokine (e.g., interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), and granulocyte colony stimulating factor ("G-CSF")), or a growth factor (e.g., growth hormone ("GH")).

[0223] Moreover, an antibody can be conjugated to therapeutic moieties such as a radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (see above for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res. 4:2483; Peterson et al., 1999, Bioconjug. Chem. 10:553; and Zimmerman et al., 1999, Nucl. Med. Biol. 26:943.

[0224] Techniques for conjugating therapeutic moieties to antibodies are well known. Moieties can be conjugated to antibodies by any method known in the art, including, but not limited to aldehyde/Schiff linkage, sulphydryl linkage, acid-labile linkage, cis-aconityl linkage, hydrazone linkage, enzymatically degradable linkage (see generally Garnett, 2002, Adv Drug Deliv Rev 53:171). Techniques for conjugating therapeutic moieties 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., 1982, Immunol. Rev. 62:119.

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[0225] Alternatively, an antibody or antibody domain can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

5.6. Production Of Multidomain Proteins

[0226] Recombinant expression of a multidomain protein, derivative, analog or fragment thereof, (*e.g.*, an antibody or fusion protein), requires construction of an expression vector containing a polynucleotide that encodes the multidomain protein (e.g., antibody, or fusion protein). Once a polynucleotide encoding a multidomain protein (e.g., antibody, or fusion protein) has been obtained, the vector for the production of the multidomain protein (e.g., antibody, or fusion protein) may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing a multidomain protein (e.g., antibody, or fusion protein) encoding nucleotide sequence are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing multidomain protein (e.g., antibody, or fusion protein) coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding a multidomain protein of the invention, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., International Publication No. WO 86/05807; International Publication No. WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody, or a polypeptide for generating an multidomain protein may be cloned into such a vector for expression of the full length antibody chain (e.g. heavy or light chain), or complete multidomain protein comprising a fusion of a non-antibody derived polypeptide and a an antibody domain.

[0227] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a multidomain. Thus, the invention includes host cells containing a polynucleotide encoding a multidomain protein of the invention, operably linked to a heterologous promoter. In specific embodiments for the expression of multidomain proteins comprising double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

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[0228] A variety of host-expression vector systems may be utilized to express the multidomain proteins of the invention (e.g., antibody or fusion protein molecules) (see, e.g., U.S. Patent No. 5,807,715). Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an multidomain protein of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing multidomain protein coding sequences; yeast (e.g., Saccharomyces Pichia) transformed with recombinant yeast expression vectors containing multidomain protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing multidomain protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing multidomain protein coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, NS0, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). In certain embodiments, bacterial cells such as Escherichia coli, or eukaryotic cells, are used for the expression of a multidomain protein which is a recombinant antibody or fusion protein molecules. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 1986, Gene 45:101; and Cockett et al., 1990, Bio/Technology 8:2). In a specific embodiment, the expression of nucleotide sequences encoding a multidomain protein of the invention (e.g., antibody or fusion protein) is regulated by a constitutive promoter, inducible promoter or tissue specific promoter.

[0229] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the multidomain protein (e.g., antibody or fusion protein) being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of a multidomain protein, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression

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vector pUR278 (Ruther et al., 1983, EMBO 12:1791), in which the multidomain protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a lac Z-fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0230] In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The multidomain protein (e.g., antibody or fusion protein) coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

[0231] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the multidomain protein (e.g., antibody or fusion protein) coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region El or E3) will result in a recombinant virus that is viable and capable of expressing the multidomain protein (e.g., antibody or fusion protein) in infected hosts (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 8 1:355-359). Specific initiation signals may also be required for efficient translation of inserted multidomain protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et al., 1987, Methods in Enzymol. 153:516-544).

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[0232] The expression of a multidomain protein (e.g., antibody or fusion protein) may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding an multidomain protein (e.g., antibody or fusion protein) include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, Proc. Nat. Acad. Sci. USA 89:5547-5551); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control

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region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al., 1999, Gen. Virol. 80:571-83); brain-derived neurotrophic factor (BDNF) gene control region which is active in neuronal cells (Tabuchi et al., 1998, Biochem. Biophysic. Res. Com. 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al., 1999, Braz J Med Biol Res 32(5): 619-631; Morelli et al., 1999, Gen. Virol. 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

[0233] Expression vectors containing inserts of a gene encoding a multidomain protein (e.g., antibody or fusion protein) can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a peptide, polypeptide, protein or a fusion protein in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding the peptide, polypeptide, protein or the fusion protein, respectively. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a nucleotide sequence encoding a multidomain protein (e.g., antibody or fusion protein) in the vector. For example, if the nucleotide sequence encoding the multidomain protein (e.g., antibody or fusion protein) is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the multidomain protein (e.g., antibody or fusion protein) insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (e.g., antibody or fusion protein) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the fusion protein in in vitro assay systems, e.g., binding with anti-bioactive molecule antibody.

[0234] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain

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inducers; thus, expression of the genetically engineered fusion protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript (e.g., glycosylation, and phosphorylation) of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, WI38, NS0, and in particular, neuronal cell lines such as, for example, SK-N-AS, SK-N-FI, SK-N-DZ human neuroblastomas (Sugimoto et al., 1984, J. Natl. Cancer Inst. 73: 51-57), SK-N-SH human neuroblastoma (Biochim. Biophys. Acta, 1982, 704: 450-460), Daoy human cerebellar medulloblastoma (He et al., 1992, Cancer Res. 52: 1144-1148) DBTRG-05MG glioblastoma cells (Kruse et al., 1992, In Vitro Cell. Dev. Biol. 28A: 609-614), IMR-32 human neuroblastoma (Cancer Res., 1970, 30: 2110-2118), 1321N1 human astrocytoma (Proc. Natl Acad. Sci. USA, 1977, 74: 4816), MOG-G-CCM human astrocytoma (Br. J. Cancer, 1984, 49: 269), U87MG human glioblastoma-astrocytoma (Acta Pathol. Microbiol. Scand., 1968, 74: 465-486), A172 human glioblastoma (Olopade et al., 1992, Cancer Res. 52: 2523-2529), C6 rat glioma cells (Benda et al., 1968, Science 161: 370-371), Neuro-2a mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1970, 65: 129-136), NB41A3 mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1962, 48: 1184-1190), SCP sheep choroid plexus (Bolin et al., 1994, J. Virol. Methods 48: 211-221), G355-5, PG-4 Cat normal astrocyte (Haapala et al., 1985, J. Virol. 53: 827-833), Mpf ferret brain (Trowbridge et al., 1982, In Vitro 18: 952-960), and normal cell lines such as, for example, CTX TNA2 rat normal cortex brain (Radany et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6467-6471) such as, for example, CRL7030 and Hs578Bst. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

[0235] For long-term, high-yield production of recombinant proteins, stable expression is often preferred. For example, cell lines which stably express a multidomain protein of the invention (e.g., antibody or fusion protein) may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter,

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enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express a multidomain protein that specifically binds to an antigen. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the activity of a multidomain protein (e.g., a polypeptide or a fusion protein) that specifically binds to an antigen.

[0236] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147) genes.

[0237] Once an multidomain protein (e.g., antibody, or a fusion protein) has been produced by recombinant expression, it may be purified by any method known in the art for purification of a protein, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

[0238] The expression levels of an multidomain protein (e.g., antibody or fusion protein) can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). For example, when a marker in the vector system expressing a multidomain protein (e.g., antibody or fusion protein) is amplifiable, increase in the level of inhibitor present in culture

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of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the multidomain protein gene, production of the multidomain protein will also increase (Crouse et al., 1983, Mol. Cell. Biol. 3:257).

[0239] The host cell may be co-transfected with two expression vectors of the invention. For example, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers, which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, a fusion protein or both heavy and light chain polypeptides. The coding sequences for the fusion protein or heavy and light chains may comprise cDNA or genomic DNA.

5.7. Preparation Of Liquid Formulations Of Multidomain Proteins

[0240] The liquid formulations of multidomain proteins, such as antibodies, can be prepared using any method known in the art. In one embodiment, a liquid preparation can be prepared by a method comprising: purifying the multidomain proteins from conditioned medium (either single lots or pooled lots of medium) and concentrating a fraction of the purified multidomain proteins to a final concentration from about 15 mg/ml, about 20 mg/ml, about 30 mg/ml, about 40 mg/ml, about 50 mg/ml, about 60 mg/ml, about 70 mg/ml, about 80 mg/ml, about 90 mg/ml, about 100 mg/ml, about 110 mg/ml, about 125 mg/ml, about 150 mg/ml, about 200 mg/ml, about 250 mg/ml, or about 300 mg/ml.

[0241] In a specific embodiment, the liquid formulations can be prepared by a method comprising concentrating a fraction containing the purified antibody or a fragment to a final antibody or fragment concentration of from about 15 mg/ml, about 20 mg/ml, about 30 mg/ml, about 40 mg/ml, about 50 mg/ml, about 60 mg/ml, about 70 mg/ml, about 80 mg/ml, about 90 mg/ml, about 100 mg/ml, about 110 mg/ml, about 125 mg/ml, about 150 mg/ml, about 200 mg/ml, about 250 mg/ml, or about 300 mg/ml using a semipermeable membrane with an appropriate molecular weight (MW) cutoff (e.g., 30 kD cutoff for whole antibody molecules and F(ab')2 fragments; and 10 kD cutoff for antibody fragments, such as Fab fragments) and difiltrating the concentrated antibody fraction into the formulation buffer using the same membrane. Conditioned medium containing antibody or a fragment thereof that specifically binds to a target antigen is subjected to CUNO filtration and the filtered antibody is subjected to HS50 cation exchange chromatography. The fraction from the HS50

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cation exchange chromatography is then subjected to rProtein A affinity chromatography followed by low pH treatment. Following low pH treatment, the antibody fraction is subject to super Q 650 anion exchange chromatography and then nanofiltration. The fraction of the antibody obtained after nanofiltration is then subjected to diafiltration to concentrate the antibody fraction into the formulation buffer using the same membrane.

[0242] The formulation buffer can comprise histidine at a concentration ranging from about 1 mM to about 100 mM, about 10 mM to about 50 mM, or about 20 mM to about 30 mM. The formulations may further comprise an amino acid at a concentration of less than 100 mM, less than 50 mM, less than 3.0 mM, less than 2.0 mM, or less than 1.8 mM. The amount of amino acid in the formulation should not cause a significant buffering in order to avoid multidomain protein precipitation at its isoelectric point. The pH of the formulation may range from about 5.0 to about 7.0, or about 5.5 to about 6.5, or about 5.8 to about 6.2, or about 6.0. To obtain an appropriate pH for a particular multidomain protein, it is generally understood that histidine (and glycine, if added) is first dissolved in water to obtain a buffer solution with higher pH than the desired pH and then the pH is brought down to the desired level by adding HCl. This way, the formation of inorganic salts (e.g., formation of NaCl when, for example, histidine hydrochloride is used as histidine and pH is raised to a desired level by adding NaOH) can be avoided.

[0243] The liquid formulations can be prepared as unit dosage forms by preparing a vial containing an aliquot of the liquid formulation for a one-time use. For example, a unit dosage per vial may contain 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 7 ml, 8 ml, 9 ml, 10 ml, 15 ml, or 20 ml of different concentrations of a multidomain protein ranging from about 15 mg/ml to about 300 mg/ml. If necessary, these preparations can be adjusted to a desired concentration by adding a sterile diluent to each vial. In a specific embodiment, the multidomain protein is an antibody that binds to a target antigen.

[0244] The liquid formulations of the present invention may be sterilized by various sterilization methods, including sterile filtration, radiation, etc. In a specific embodiment, the difiltrated antibody formulation is filter-sterilized with a presterilized 0.2 or 0.22-micron filter.

5.8. Preparation Of Lyophilized Formulations Of Multidomain Proteins

[0245] Lyophilization is a freeze drying process that is often used in the preparation of pharmaceutical products to preserve their biological activity. The liquid composition is

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prepared, then lyophilized to form a dry cake-like product. The process generally involves drying a previously frozen sample in a vacuum to remove the ice, leaving the non- water components intact, in the form of a powdery or cake-like substance. The lyophilized product can be stored for prolonged periods of time, and at elevated temperatures, without loss of biological activity, and can be readily reconstituted into a particle-free solution by the addition of an appropriate diluent. An appropriate diluent can be any liquid which is biologically acceptable and in which the lyophilized powder is completely soluble. Water, particularly sterile, pyrogen-free water, is a preferred diluent, since it does not include salts or other compounds which may affect the stability of the multidomain protein. The advantage of lyophilization is that the water content is reduced to a level that greatly reduce the various molecular events which lead to instability of the product upon long-term storage. The lyophilized product is also more readily able to withstand the physical stresses of shipping. The reconstituted product is preferably substantially particle free, thus it can be administered without prior filtration.

[0246] The following criteria are important in developing stable lyophilized protein products. Protein unfolding during lyophilization should be minimized. Various degradation pathways should be minimized. Residual moisture should be low. A strong and elegant cake structure should be obtained. A reconstitution time 15 should be short, for example, less than 60 minutes, or less then 40 minutes, or less then 20 minutes, or less then 10 minutes, or less then 5 minutes, or less than 2 minutes, or less than 1 minute.

[0247] The protein in the pre-lyophilized formulation has a high concentration of at least 5 mg/ml, or at least 10 mg/ml, or at least 25 mg/ml, or at least 50 mg/ml or greater. The ph of the formulation may range from about 5.0 to about 7.0, or about 5.5 to about 6.5, or about 5.8 to about 6.2, or about 6. Examples of buffers that control the ph in this range include succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. Histidine is a preferred buffer for subcutaneous, intramuscular and peritoneal injection. A specific buffer contains about 5-30 mm histidine.

[0248] A surfactant may be added to the protein formulation. exemplary surfactants include nonionic surfactants such as polysorbates (*e.g.* polysorbates 20, 80, such as tween 20, 10 tween 80) or poloxamers (*e.g.* poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated protein and/or minimizes the formation of particulates in the formulation and/or reduces protein adsorption onto the container. The surfactant also reduces the reconstitution time of the lyophilized formulation. For example,

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the surfactant is present in the formulation in an amount from about 0.001% to about 0.5%, or from about 0. 005% to about 0.1 % or from about 0.01% to about 0.05%.

[0249] Lyophilized formulations may optional contain one or more of the following, an excipient, such as saccharides, polyols and sugar alcohols, a bulking agent that provides good lyophilized cake properties, such as serine, glycine, mannitol, and tonicity modifiers such as salts (*e.g.*, NaCl, KCl, $MgCl_2$, $CaCl_2$).

5.9. Administration Of Formulations Of Multidomain Proteins

[0250] The formulated protein drug can be administrated to a subject in an effective amount for treating or preventing a disease condition in the subject. The subject is preferably a mammal such as non-primate (*e.g.*, cows, pigs, horses, cats, dogs, rats etc.) and a primate (*e.g.*, monkey such as a cynomolgous monkey and a human). In a specific embodiment, the subject is a human.

[0251] Various delivery systems are known and can be used to administer the formulated drug. Methods of administering formulations include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural administration, topical administration, pulmonary administration, and mucosal administration (*e.g.*, intranasal and oral routes). In a specific embodiment, liquid formulations, including reconstituted lyophilized formulations, of a therapeutic protein are administered intramuscularly, intravenously, subcutaneously or , intramuscularly. The formulations may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, pulmonary administration can be employed, *e.g.*, by use of an inhaler or nebulizer.

[0252] The formulation of the present invention can be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of multidomain protein. It is contemplated that the formulations of the present invention are in a hermetically sealed container indicating the quantity and concentration of multidomain protein. in certain embodiments, the formulation of the present invention is supplied in a hermetically sealed container at least 15 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml, 70 mg/ml, 80 mg/ml, 90 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, 250 mg/ml, or 300 mg/ml, in a quantity of about 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 7 ml, 8 ml, 9 ml, 10 ml, 15 ml, or 20 ml.

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[0253] The amount of the formulations of the present invention which will be effective in the treatment, prevention, management or amelioration of a disease, one or more symptoms thereof, or an undesirable condition associated with the disease, can be determined by standard clinical techniques. For example, the dosage of a drug formulation which will be effective in the treatment, prevention, management or amelioration of a disease can be determined by administering the formulation to an animal model (*e.g.*, a cotton rat or monkey) and measuring the serum titer of the active protein, *e.g.*, the serum titer of the multidomain protein. Accordingly, a dosage of the formulation that results in a serum titer of at least 1 μg/ml, at least 2 μg/ml, at least 5 μg/ml, at least 10 μg/ml, at least 20 μg/ml, at least 25 μg/ml, at least 35 μg/ml, at least 40 μg/ml, at least 50 μg/ml, at least 75 μg/ml, at least 100 μg/ml, at least 125 μg/ml, at least 150 μg/ml, at least 200 μg/ml, at least 250 μg/ml, at least 300 μg/ml, at least 350 μg/ml, at least 400 μg/ml, or at least 450 μg/ml can be administered to a human for the treatment, prevention, management or amelioration of a disease. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. In specific embodiments, the multidomain protein is an antibody that bind to a target antigen

[0254] The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease, 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 (*e.g.*, the cotton rat or cynomolgous monkey) test systems.

[0255] For antibodies, proteins, polypeptides, peptides and fusion proteins, the dosage administered to a patient may be about 0.1 mg/kg to 30 mg/kg of the patients body weight. For example, generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage, volume and frequency of administration of liquid formulations, including reconstituted lyophilized formulations, of the present invention may be reduced by increasing the concentration of an antibody or a fragment thereof in the formulations, increasing affinity and/or avidity of the antibody or a fragment thereof, and/or increasing the half-life of the antibody or a fragment thereof.

[0256] Exemplary doses of a small molecule include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per

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kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram).

6. EXAMPLES

[0257] The following examples are presented by way of illustration of the present invention, and is not intended to limit the present invention in any way. In the examples, to show the structural relationships among the antibodies, a naming scheme illustrated in Fig. 23 is used. In the naming scheme, the first 3 characters identify the antigen binding domain: the capital letter designates the target, the lower case letter designates a particular epitope of the target, the number designates the variant of the antibody (for simplicity reasons, number 0 is often omitted). The last 4 characters identify the Fc domain: the first 3 characters identify the source and subclass of the Fc domain, the last lower case letter designates the Fc variant. The example in FIG. 23, Aa1-hG1a, thus identifies the "1" variant of an antibody that binds epitope "a" of target "A" and has variant "a" of a human gamma-1 (hG1) as its Fc domain. Such a naming scheme allows easy determination, *e.g.*, that antibodies Aa-hG1 and Aa1-hG1 are different variants of an antibody that both bind to the same epitope of the same target and have the same Fc domain, or that antibodies Aa-hG1 and Ab-hG1 are different antibodies that bind to different epitopes of the same target and have the same Fc region, and so on.

6.1. EXAMPLE 1: Contribution Of Fab And Fc Domains To Tm Values And Stability

[0258] The Fab and Fc fragments of Ca-hG1 and Ba-hG1 were generated in order to learn more about the properties of the antibody Components. One long-term goal in studies of intact Mabs and their fragments is to understand the relationship between stability and structure. Currently it is known that Ca-hG1 is more stable with respect to aggregation when compared to Ba-hG1. This stability is further confirmed using the battery of analytical stability indicating assays. This stability difference offers an opportunity to compare properties of each antibody and to determine if there are measurable differences that can be exploited to understand the stability of these specific Mabs and more broadly applied to additional antibodies.

[0259] Fab and Fc domains were generated from full Mabs Ca-hG1 and Ba-hG1 using papain. A commercial kit from Pierce (Immunopure Fab Preparation Kit Pierce product # 44885: Immunopure IgG Binding Buffer, Immunopure IgG Elution Buffer,

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Affinitypak Immobilized Protein A Column, Immobilized Papain, Cysteine Monohydrochloride, Phosphate Buffer, and Serum Separators) was used to digest the intact antibodies. Ca-hG1 was successfully digested using the method described in the kit. Ba-hG1 required an alternate chromatography step. For Ba-hG1, both the Fab and Fc bound to the protein A column. Purification of these components required anion exchange chromatography (Hitrap DEAE FF 5 ml –Amersham Product #17-5154-01). In addition to chromatography optimization, enzymology was optimized to achieve the best cleavage of the Mab in a reasonable time.

[0260] Fab and Fc domains were generated from Ca-hG1 using the following steps: A) adding antibody to papain and incubating overnight at 37°C, ~ 10 mg of IgG per digestion; B) separating crude digest from immobilized enzyme; C) applying digest to protein A column; D) eluting the Fab fragment in unretained fraction at pH-8.0; E) eluting the Fc fragment at pH-3.0; and F) dialyzing the fragments into a required buffer.

[0261] Fab and Fc domains were generated from Ba-hG1 using the above procedure with the following modification: Using double enzyme to Mab ratio, increasing agitation, increasing incubation time to 24 hrs, omitting protein A column, buffering exchange digested solution into equilibration buffer for DEAE column (Start Buffer: 10 mM NaCl, 10 mM Tris pH-7.8, Elution Buffer: 100 mM NaCl, 10 mM Tris pH-7.8). The Fab portion of the antibody was in the unretained fraction which was eluted during sample loading. The Fc fragment was eluted with the 100 mM NaCl buffer. Table 1 summarizes the Fab and Fc fragments obtained by the procedures.

Table 1. Fab And Fc Obtained

Fragment	Ca-hG1	Ba-hG1
Fab	Purified	No Recovery
Fc	Purified	Fc+Fab Mix

[0262] Differential Scanning Calorimetry (DSC) was used to examine the melting curve of the full length monoclonal antibody (Mab) Ca-hG1 (FIG. 1, Top). Deconvolution of the thermogram of the full length Mab reveals that at least three separate Tm values (Tm1 = 69°C, Tm2 = 83°C And Tm3 = 87°C) make up the curve (FIG. 2). Fab and Fc domain fragments were generated from Ca-hG1 and the purified fragments were analyzed individually by DSC (FIG. 1, bottom). The results show that individual Tm values in a full antibody may be assigned to individual domains with the largest peak generated by the Fab domain (FIG. 1 bottom, also see FIG. 10 bottom). Analysis of the peaks generated by the Fc

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domain indicate that the Tm of the C_H2 region is represented by the first peak in the Fc DSC profile and the Tm of the C_H3 region is represented by the second peak (FIG. 1 bottom, also see FIG. 10 bottom).

[0263] The reversibility of melting was examined at two different temperatures for the full length Ca-hG1 Mab (FIGS. 3 and 4), The Fab domain (FIGS. 5 and 6) and the Fc domain (FIGS. 7 and 8). The results show that instability of the full antibody is a result of the instability of the Fab domain.

[0264] The DSC profiles of an unrelated full length Mab, Ba-hG1, were also measured and compared to Ca-hG1 (FIG. 9). To determine which domain contributes the most to the differences in the Tm profile, The DSC profiles of the Fab and Fc domains of Ba-hG1 were determined individually. In FIG. 10, the top panel shows the DSC profile for the intact Ba-hG1 antibody and the bottom panel shows the profiles for the Fc and Fab domains. As was seen for Ca-hG1, the largest peak is generated by the melting of the Fab domain. As expected, the Fc region generated two smaller peaks which correspond to those seen for the identical Fc region isolated from Ca-hG1 (compare FIG. 1 and 10, bottom panels). Deconvolution of the full antibody DSC profile indicates that at least three separate Tm values (Tm1 = 71°C, Tm2 = 82°C And Tm3 = 93°C) make up the curve of full length Ba-hG1 (FIG. 11). The reversibility of melting was examined for the full length Ba-hG1 Mab (FIGS. 12 and 13), The Fab domain (FIGS. 14 and 15) and the Fc domain (FIG. 16). The results also indicate that instability of the full antibody is a result of the instability of the Fab domain.

[0265] A comparison of the DSC profiles of Ba-hG1 and Ca-hG1 shows that the Fab domain contributes the most to the difference between the profiles for the two different Mab molecules (compare FIG. 1, bottom panel and FIG. 9, bottom panel). The results show that Ba-hG1 is less stable than Ca-hG1 because the Fab domain of Ba-hG1 is less stable than the Fab domain of Ca-hG1. Thus, the stability of an antibody may be predicted by evaluating the stability of the Fab domain.

6.2. EXAMPLE 2: Examination Of The Contribution Of The Variable Domain To Tm And pI Values

[0266] Fab fragments from 6 individual antibodies, several of which recognize the same epitope (Ca-hG1, Ca1-hG1 and Ca1-hG1a) were isolated. The Tm and pI for each was determined. The Fab fragments from Ca-hG1 and Ca1-hG1 differ by only about 13 amino acids, while Ca1-hG1 and Ca1-hG1a differ by only 3 amino acids. These three highly related

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molecules show only small differences in their pI and Tm values. In contrast, the completely unrelated molecules, Aa-hG1, Ba-hG1 and Da-hG1, show very different profiles (FIG. 17).

[0267] To examine the contribution of the Fab region to Tm and pI values in the context of a full length antibody, 18 individual Fab clones isolated from a single phage display library were converted into full length IgG1 and purified from transient transfections. These molecules differ only in their variable regions. The subsequent analysis of these clones demonstrate that they have a wide range of characteristics. For example, they exhibit dissociation constants (Kd) between a high of about 330 nM to a low of just 22 nM (data not shown). The Tm values of the Fab fragments, determined by DSC analysis of the intact antibodies, range from a low of just about 70°C to a high of about 90°C (FIGS. 18B and 18C). pI values of the intact antibodies also showed a wide range with the antibodies having pI values from 7.8 – 9.0 (FIGS. 18A and 18C).

[0268] FIG. 19 shows Tm and pI values of an additional panel of antibodies that bind to a variety of different epitopes present on a single protein as well as the previously analyzed Ca1-hG1 and two chimeric antibodies (Ha-hG1, Ia-hG1) generated by combining the heavy and light chains from two different antibodies. These antibodies also have a common Fc domain and vary only in their Fab domains. As was seen above, the different Fab domains result in a wide range of both pI and Tm values. In contrast, FIG. 20 shows the Tm and pI values of a panel of antibodies which differ by only a few amino acids in the hinge domain. These hinge variants, which have identical Fab domains, vary little in either their pI or Tm. Note that for the hinge variants for which a pI was determined, the amino acid changes made did not alter the net charge based on amino acid sequence.

[0269] FIG. 21 shows the viscosities of a 100 mg/mL solution of Ca-hG1, Ca1-hG1, Ba-hG1 and Da-hG1 as a function of temperature. It can be seen that the antibody with the lowest pI, Da-hG1, had the highest viscosity under the conditions measured. Also shown is the viscosity of Ba-hG1 as a function of temperature at increasing concentrations (100, 126, 138 and 150 mg/mL). The higher the concentration the higher the viscosity of the antibody solution. Accordingly, for the formulation of high concentration liquid formulations it is generally desirable to use an antibody with a lower inherent viscosity. These studies indicate that the pI of an antibody provides a measure indicative of the viscosity of the antibody in solution.

[0270] FIG. 22A shows the aggregation rates of the Ba-hG1, Ca-hG1, Ca1-hG1 and Da-hG1 antibodies at ~40°C as a function of the Tm of the Fab. It can be seen that the

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DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15260888 Document Date: 09/09/2016

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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: September 9, 2016
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

Application No.: Not Yet Assigned

Confirmation No.: N/A

Filed: Concurrently Herewith

Art Unit: N/A

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

Examiner: Not Yet Assigned

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Dear Sir:

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please insert the following paragraphs after the title:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application No. 15/148,839, filed May 6, 2016, which is a continuation of U.S. Patent Application No. 13/426,973, filed March 22, 2012, which is a continuation of U.S. Patent Application No. 12/225,040, filed on May 13, 2009, which is a 35 U.S.C. 371 national stage filing of International Application No. PCT/US2007/006606, filed March 15, 2007. The contents of the aforementioned applications are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 8, 2016, is named AXJ_114BUSCN3_Seq_Listing.txt and is 10,483 bytes in size.

REMARKS

Amendments to the Specification

Applicant has amended the specification at page 1 to update the related applications and Sequence Listing sections. *No new matter has been added.*

CONCLUSION

If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 217-4630.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN3.

Dated: September 9, 2016

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630 (617) 742-4214 (Fax)
Attorney/Agent For Applicant

Electronic Patent Application Fee Transmittal	
Application Number:	
Filing Date:	
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Filer:	Jill R. Gorny Sloper/Danielle Gamache
Attorney Docket Number:	AXJ-114BUSCN3

Filed as Large Entity

Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
Request for Prioritized Examination	1817	1	4000	4000
Pages:				
Claims:				
Miscellaneous-Filing:				
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140

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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**5740**

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Electronic Acknowledgement Receipt	
EFS ID:	26877080
Application Number:	15260888
International Application Number:	
Confirmation Number:	2558
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Danielle Gamache
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Receipt Date:	09-SEP-2016
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Time Stamp:	14:43:55
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$5740
RAM confirmation Number	1442
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 CFR 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 CFR 1.17 (Patent application and reexamination processing fees)	

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	AXJ_114BUSCN3_ADS.pdf	62210 2916f90aa33954f60b30947edfad8ea13b903717	no	9
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
2	Drawings-only black and white line drawings	AXJ_114BUSCN3_Figures.pdf	990652 d39f7fb9a45e3f53a1be19b0561ddf1307db5f45	no	4
Warnings:					
Information:					
3	Oath or Declaration filed	AXJ_114BUSCN3_Dec.pdf	2443083 aec88d61e9a45b53a97b118d2d8ea79a0fe39d07	no	3
Warnings:					
Information:					
4	Power of Attorney	AXJ_114BUSCN3_POA.pdf	210805 eaf70c0c70a173443ced1184ec018edf9d87b414	no	2
Warnings:					
Information:					
5	CRF Statement Paper and CRF are the same	AXJ_114BUSCN3_Statement_re_SL.pdf	110908 e28e603ef56e1921be272a74dfd10b2cb7ba9f9f	no	2
Warnings:					
Information:					
6	TrackOne Request	AXJ_114BUSCN3_TrackOneReq.pdf	109652 1d2fb0b348de3c9308aeb802a89e50d34d181545	no	1
Warnings:					

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Information:					
7		AXJ_114BUSCN3_Specification.pdf	4029306 c54764b0bad6f0be2b38bf47d9a2020365ea2503	yes	47
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	45	
	Claims		46	46	
	Abstract		47	47	
Warnings:					
Information:					
8	Sequence Listing (Text File)	AXJ_114BUSCN3_Seq_Listing.txt	10483	no	-
Warnings:					
Information:					
9		AXJ_114BUSCN3_PrelimAmdt.pdf	135323 82e536fdb5dc6640f139c3b76111521ca2884073	yes	3
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Preliminary Amendment		1	1	
	Specification		2	2	
	Applicant Arguments/Remarks Made in an Amendment		3	3	
Warnings:					
Information:					
10	Fee Worksheet (SB06)	fee-info.pdf	38607 5c46badbaf199fae985904b6fbac66821317eee6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	8141029		

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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Leonard		BELL	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Woodbridge	State/Province	CT	Country of Residence	US

Mailing Address of Inventor:

Address 1	59 Tumblebrook Road		
Address 2			
City	Woodbridge	State/Province	CT
Postal Code	06525	Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Russell	P.	ROTHER	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Oklahoma City	State/Province	OK	Country of Residence	US

Mailing Address of Inventor:

Address 1	10325 Forest Meadow Drive		
Address 2			
City	Oklahoma City	State/Province	OK
Postal Code	73151	Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

☐ **An Address is being provided for the correspondence Information of this application.**			
Customer Number	133485		
Email Address	IPBoston.Docketing@nelsonmullins.com	Add Email	Remove Email

Application Information:

Title of the Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT		
Attorney Docket Number	AXJ-114BUSCN3	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	4	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	133485		

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	15/148839	2016-05-06
Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
15/148839	Continuation of	13/426973	2012-03-22
Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13/426973	Continuation of	12/225040	2009-05-13
Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
12/225040	a 371 of international	PCT/US2007/006606	2007-03-15

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Alexion Pharmaceuticals, Inc.

Mailing Address Information For Applicant:

Address 1	100 College St.		
Address 2			
City	New Haven	**State/Province**	CT
Country	US	Postal Code	06510
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Jill Gorny Sloper, Esq./			Date (YYYY-MM-DD)	2016-09-09
First Name	Jill Gorny	Last Name	Sloper	Registration Number	60760

Additional Signature may be generated within this form by selecting the Add button.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	AXJ-114BUSCN3
	Application Number	
Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

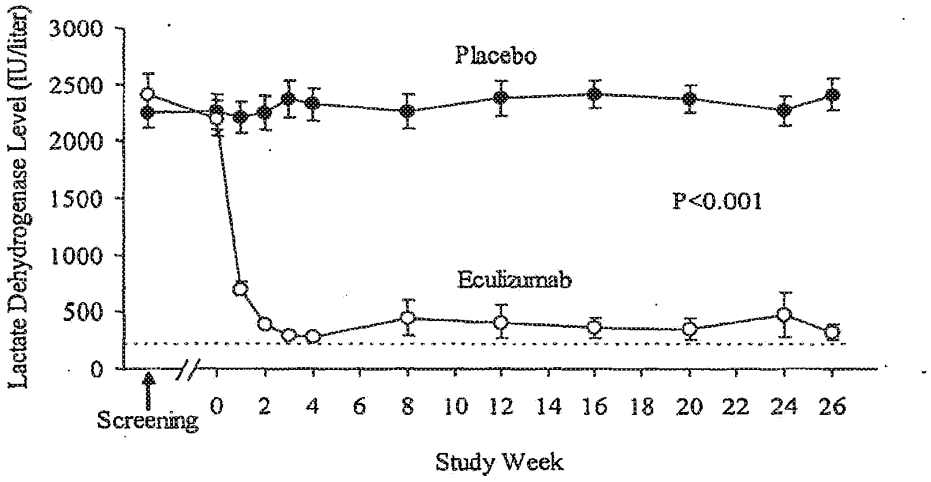

Lactate Dehydrogenase Level (IU/liter)
3000
2500
2000
1500
1000
500
0
Placebo
P<0.001
Eculizumab
Screening
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Study Week

FIGURE 1A

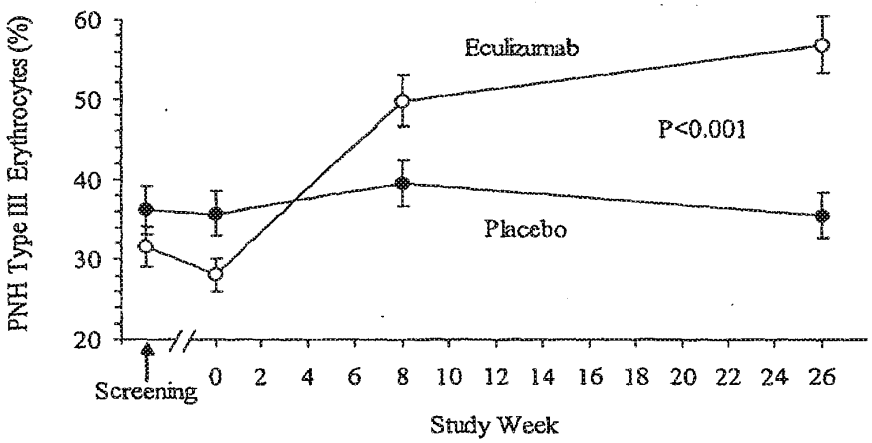


FIGURE 1B

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Page 10061

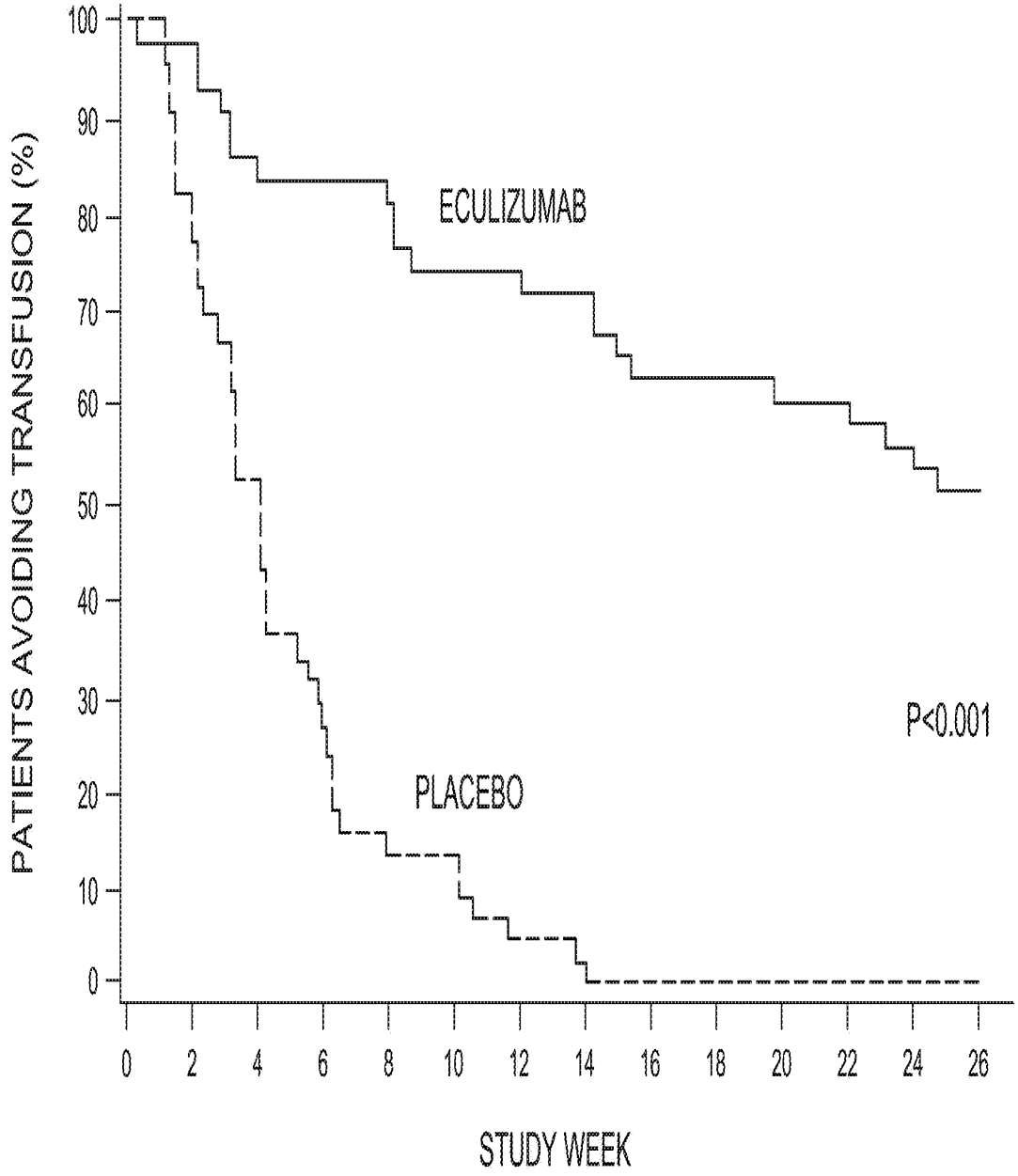


FIGURE 2

AXJ-114BUSCN3

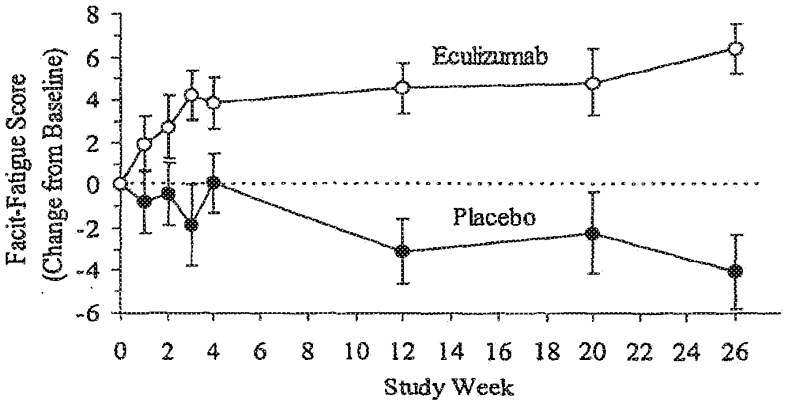


FIGURE 3

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

As the below named inventor, I hereby declare that:

This declaration is directed to:
- [ ] The attached application, or
- [x] United States application or PCT international application number 15/148,839 filed on 05/06/2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Leonard Bell Date (Optional): _____

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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Page 10064

Doc code: Oath
Document Description: Oath or declaration filed

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SUBSTITUTE STATEMENT IN LIEU OF AN OATH OR DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (35 U.S.C. 115(d) AND 37 CFR 1.64)

Title of Invention	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

This statement is directed to:

[ ] The attached application,

OR

[X] United States application or PCT international application number 15/148,839 filed on 05/06/2016.

LEGAL NAME of inventor to whom this substitute statement applies:

(*E.g.*, Given Name (first and middle (if any)) and Family Name or Surname)

Russell P. ROTHER

Residence (except for a deceased or legally incapacitated inventor):

City	State	Country
Oklahoma City	OK	United States of America

Mailing Address (except for a deceased or legally incapacitated inventor):

10325 Forest Meadow Drive

City	State	Zip	Country
Oklahoma City	OK	73151	United States of America

I believe the above-named inventor or joint inventor to be the original inventor or an original joint inventor of a claimed invention in the application.

The above-identified application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this statement is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Relationship to the inventor to whom this substitute statement applies:

[ ] Legal Representative (for deceased or legally incapacitated inventor only),

[X] Assignee,

[ ] Person to whom the inventor is under an obligation to assign,

[ ] Person who otherwise shows a sufficient proprietary interest in the matter (petition under 37 CFR 1.46 is required), or

[ ] Joint Inventor.

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PTO/SB/AIA02 (07-13)
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SUBSTITUTE STATEMENT

Circumstances permitting execution of this substitute statement:

- [ ] Inventor is deceased,
- [ ] Inventor is under legal incapacity,
- [ ] Inventor cannot be found or reached after diligent effort, or
- [x] Inventor has refused to execute the oath or declaration under 37 CFR 1.63.

If there are joint inventors, please check the appropriate box below:

- [x] An application data sheet under 37 CFR 1.76 (PTO/AIA/14 or equivalent) naming the entire inventive entity has been or is currently submitted.

OR

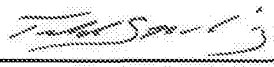
- [ ] An application data sheet under 37 CFR 1.76 (PTO/AIA/14 or equivalent) has not been submitted. Thus, a Substitute Statement Supplemental Sheet (PTO/AIA/11 or equivalent) naming the entire inventive entity and providing inventor information is attached. See 37 CFR 1.64(b).

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

PERSON EXECUTING THIS SUBSTITUTE STATEMENT:

Name: Todd Spalding	Date (Optional):

Signature: 

APPLICANT NAME AND TITLE OF PERSON EXECUTING THIS SUBSTITUTE STATEMENT:

If the applicant is a juristic entity, list the applicant name and the title of the signer:

Applicant Name: Alexion Pharmaceuticals, Inc.

Title of Person Executing This Substitute Statement: Vice President, Head of Intellectual Property

The signer, whose title is supplied above, is authorized to act on behalf of the applicant.

Residence of the signer (unless provided in an application data sheet, PTO/AIA/14 or equivalent):

City	State	Country

Mailing Address of the signer (unless provided in an application data sheet, PTO/AIA/14 or equivalent)

100 College St.

City: New Haven	State: CT	Zip: 06510	Country: US

Note: Use an additional PTO/AIA/02 form for each inventor who is deceased, legally incapacitated, cannot be found or reached after diligent effort, or has refused to execute the oath or declaration under 37 CFR 1.63.

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Doc Code: PA..
Document Description: Power of Attorney

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TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Leonard BELL
Title	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	AXJ-114BUSCN3

SIGNATURE of Applicant or Patent Practitioner

Signature	/Jill Gorny Sloper, Esq./	Date (Optional)	September 9, 2016
Name	Jill Gorny Sloper, Esq.	Registration Number	60,760
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of 1 forms are submitted.

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: September 9, 2016 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Samsung Bioepis Exhibit No. 1002
Page 10067

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 133485

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

[X] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name	Jane E. Remillard, Esq. NELSON MULLINS RILEY & SCARBOROUGH LLP				
Address	One Post Office Square				
City	Boston	State	MA	Zip	02109-2127
Country	US				
Telephone	(800) 237-2000	Email	IPBoston.Docketing@nelsonmullins.com		

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Alexion Pharmaceuticals, Inc.

[ ] Inventor or Joint Inventor (title not required below)

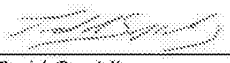
[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	07/20/15
Name	Todd Spalding		
Title	Vice President, Head of Intellectual Property		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted.

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Dated: September 9, 2016
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

Application No.: Not Yet Assigned

Confirmation No.: N/A

Filed: Concurrently Herewith

Art Unit: N/A

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

Examiner: Not Yet Assigned

MS Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT PURSUANT TO 37 CFR 1.821

Dear Sir:

Submitted herewith in connection with the above-referenced patent application and in full compliance with 37 C.F.R. §§1.821-1.825 is a computer readable copy and paper copy of the Sequence Listing (filed together as a .txt file via the United States Patent Office's Electronic Filing System).

I hereby state that I have reviewed the paper copy of the Sequence Listing, as required by 37 CFR 1.821(c), and have reviewed the computer readable form of the Sequence Listing, as required by 37 CFR 1.821(e), and that the content of the paper and computer readable copies for the above-referenced Patent application are the same as required by 37 CFR 1.821(f) (note that these documents are submitted as one electronic file). No new matter has been added to the Sequence Listing.

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Dated: September 9, 2016

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, Esq./
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY &
SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630 (617) 742-4214 (Fax)
Attorney/Agent For Applicant

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Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Leonard BELL	Nonprovisional Application Number (if known):	Not Yet Assigned
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.
2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.
3. The applicable box is checked below:

I. [x] **Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)**

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. [ ] **Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)**

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature	/Jill Gorny Sloper, Esq./	Date	September 9, 2016
Name (Print/Typed)	Jill Gorny Sloper, Esq.	Practitioner Registration Number	60,760

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

[ ] *Total of 1 forms are submitted.

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: September 9, 2016 Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

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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, compelstatin, 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 intravasoular 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.html) 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., Ch3). 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/1/1/79; 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 meningitidas*. 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.

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

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

Treatment Group	LDH Category	Change of FACIT from Baseline		p Value
		< 4	>= 4	
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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Ref Type: Abstract

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SEQUENCES

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SLSLGK

SEQ ID NO: 3 – Eculizumab V_L

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SEQ ID NO: 5 – Eculizumab CDRH1

NYWIQ

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SEQ ID NO: 6 – Eculizumab CDRH2
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SEQ ID NO: 7 – Eculizumab CDRH3
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SEQ ID NO: 8 – Eculizumab CDRL1
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SEQ ID NO: 9 – Eculizumab CDRL2
GATNLAD

SEQ ID NO: 10 – Eculizumab CDRL3
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WHAT IS CLAIMED IS:

1. A method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH) comprising administering to the patient a pharmaceutical composition comprising an antibody that binds C5, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

2. The method of claim 1, wherein the pharmaceutical composition is administered by intravenous infusion.

3. The method of claim 1, wherein the antibody is administered to the patient at a dosage level of between 5 mg per kg and 50 mg per kg per patient per treatment.

4. The method of claim 1, wherein the pharmaceutical composition is in a single unit dosage form.

5. The method of claim 4, wherein the single unit dosage form is a 300 mg single unit dosage form.

6. The method of claim 1, wherein the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution.

7. The method of claim 1, wherein the patient is anemic.

8. The method of claim 1, wherein the patient is dosed as follows: 600 mg of the antibody via intravenous infusion every 7±1 days for 4 doses; followed by 900 mg of the antibody via intravenous infusion 7±1 days later; followed by a maintenance dose of 900 mg of the antibody via intravenous infusion every 14±2 days.

9. The method of claim 1, wherein administration of the antibody results in an immediate and sustained decrease in mean levels of lactate dehydrogenase (LDH).

10. The method of claim 9, wherein the immediate decrease occurs within one week of administration of the antibody.

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TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

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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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/260,888

APPLICATION AS FILED - PART I

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INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 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).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1600

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

Samsung Bioepis Exhibit No. 1002
Page 10128

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/260,888	09/09/2016	1677	1600	AXJ-114BUSCN3	10	1

CONFIRMATION NO. 2558

133485
Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

FILING RECEIPT

OC000000085897308

Date Mailed: 09/20/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Leonard BELL, Woodbridge, CT;
Russell P. Rother, Oklahoma City, OK;

Applicant(s)

Alexion Pharmaceuticals, Inc., New Haven, CT;

Power of Attorney: The patent practitioners associated with Customer Number 133485

Domestic Priority data as claimed by applicant

This application is a CON of 15/148,839 05/06/2016
which is a CON of 13/426,973 03/22/2012
which is a CON of 12/225,040 05/13/2009 ABN
which is a 371 of PCT/US2007/006606 03/15/2007

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/19/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/260,888**

Projected Publication Date: 12/29/2016

Non-Publication Request: No

Early Publication Request: No

Title

TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

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No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/260,888	09/09/2016	Leonard BELL	AXJ-114BUSCN3	2558

133485 7590 09/29/2016

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston, MA 02109-2127

EXAMINER	
ROGERS, JAMES L	

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
09/29/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipboston.docketing@nelsonmullins.com
chris.schlauch@nelsonmullins.com
ipqualityassuranceboston@nelsonmullins.com

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Commissioner for Patents
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Doc Code:
TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.:15/260,888

1. THE REQUEST FILED September 9, 2016 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination
A. ☒ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Kimberly Inabinet at 571-272-4618.

/Kimberly Inabinet/ Paralegal Specialist, Office of Petitions

U.S. Patent and Trademark Office
PTO-2298 (Rev. 02-2012)

Samsung Bioepis Exhibit No. 1002
Page 10133

Office of Petitions: Routing Sheet



Application No. 15/260,888

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

Samsung Bioepis Exhibit No. 1002
Page 10134

Office of Petitions: Decision Count Sheet

Mailing Month

Application No. 15260888

15260888

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456

For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: Kimberly Inabinet

Count (1) - Palm Credit 15/260,888

Decision: GRANT

FINANCE WORK NEEDED

☐ Select Check Box for YES

GRANT

Decision Type: 643 - Track One request

643

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED

☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED

☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

Printed on: *9/26/2016*

Office of Petitions Internal Document - Ver. 5.0

Samsung Bioepis Exhibit No. 1002
Page 10135

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: October 21, 2016
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, /

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

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

In compliance with 37 C.F.R. § 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached PTO/SB/08. It is respectfully requested that the documents listed on the PTO/SB/08 be expressly considered by the Examiner during the prosecution of this application, and that the documents be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

The present application is a continuation of U.S. Application No. 15/148,839, filed May 6, 2016 (Atty. Docket No. AXJ-114BUSCN2). For the Examiner's convenience in reviewing this continuation application, Applicant submits a consolidated PTO Form SB/08, listing all references cited during the prosecution of the parent applications [U.S. Application No. 15/148,839, filed May 6, 2016 (Atty. Docket No. AXJ-114BUSCN2), U.S. Application No. 13/426,973, filed March 22, 2012 (Atty. Docket No. AXJ-114BUSCN), and U.S. Application No. 12/225,040, filed May 13, 2009 (Atty. Docket No. AXJ-0114BUS)].

Samsung Bioepis Exhibit No. 1002
Page 10136

Copies of the references previously cited by or submitted to the Office in the parent applications are not enclosed, but will be provided upon request, in accordance with 37 CFR §1.98(d). In addition, Applicant submits herewith copies of foreign patents and non-patent literature references, but has not included copies of U.S. patents and U.S. patent applications, in accordance with 37 C.F.R. § 1.98(a)(2)(i)-(iv).

Additionally, Applicant calls to the attention of the Examiner the following Applications and Office Actions cited therein:

Applications				
Examiner's Initials	*Serial No.*	*Filing Date*	*First Named Inventor*	*Docket No.*
	15/284,015	10-03-2016	Leonard Bell	AXJ-114BUSCN4
	15/148,839	05-06-2016	Leonard Bell	AXJ-114BUSCN2
	13/426,973	03-22-2012	Leonard Bell	AXJ-114BUSCN
	12/225,040	05-13-2009	Leonard Bell	AXJ-114BUS
	12/621,792	11-19-2009	Leonard Bell	AXJ-114DV
	10/771,552	02-03-2004	Leonard Bell	AXJ-114RCE
	11/050,543	02-03-2005	Leonard Bell	AXJ-114CPRCE
	11/595,118	11-08-2006	Leonard Bell	AXJ-114CP2
	12/902,617	10-12-2010	Leonard Bell	AXJ-114CPCN
	13/335,518	12-22-2011	Leonard Bell	AXJ-114CPCN2RCE

Office Actions (copies enclosed)			
Examiner's Initials	Serial No.	Date Mailed from USPTO	Examiner
	15/148,839	08-16-2016	J. Rogers
	13/426,973	01-27-2014	J. Rogers
	13/426,973	05-31-2013	J. Rogers
	13/426,973	08-20-2012	J. Rogers
	12/225,040	09-22-2011	J. Rogers
	12/225,040	06-01-2011	J. Rogers
	12/225,040	04-11-2011	J. Rogers
	12/621,792	09-30-2010	P. Gambel
	10/771,552	12-22-2008	F. Vandervegt
	10/771,552	03-21-2008	F. Vandervegt
	10/771,552	08-10-2007	F. Vandervegt
	10/771,552	02-26-2007	F. Vandervegt
	10/771,552	09-26-2006	F. Vandervegt

	11/050,543	07-09-2010	P. Gambel
	11/050,543	04-02-2008	F. Vandervegt
	11/050,543	10-10-2007	F. Vandervegt
	11/050,543	06-11-2007	F. Vandervegt
	11/595,118	01-21-2011	P. Gambel
	11/595,118	10-05-2010	P. Gambel
	11/595,118	07-07-2010	P. Gambel
	11/595,118	04-03-2009	F. Vandervegt
	12/902,617	06-23-2011	P. Gambel
	12/902,617	04-04-2011	P. Gambel
	13/335,518	08-22-2016	P. Gambel
	13/335,518	09-12-2014	P. Gambel
	13/335,518	02-06-2014	P. Gambel
	13/335,518	11-07-2013	P. Gambel

The Examiner is requested to review the file histories of these applications, including cited references, Office Actions, Responses, etc., and is asked to contact Applicant's Attorney if the Examiner would like the Applicant to supply copies of any or all of the information included in any of these applications. For any of these applications, if Applicant's Attorney is not contacted by the Examiner with such a request, then it will be concluded that the Examiner has reviewed or will review the file content of these applications.

Applicant respectfully requests that the Examiner initial the blank columns next to the cited Applications and Office Actions, to indicate that the information has been considered by the Examiner. Alternatively, Applicant requests that the Examiner insert the phrase, "All references considered except where lined through," on each page of the Information Disclosure Statement, along with the Examiner's initials.

The filing of this Information Disclosure Statement is not to be interpreted as a representation that the cited documents are material, that an exhaustive search has been conducted, or that no other relevant information exists. Nor shall the citation of any documents herein be construed *per se* as a representation that such document is prior art. Moreover, Applicant understands the Examiner will make an independent evaluation of the cited documents.

This Information Disclosure Statement is filed within three months of the U.S. filing date (37 C.F.R. § 1.97(b)(1)). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 12-0080, under Order No. AXJ-114BUSCN3.

Dated: October 21, 2016

Respectfully submitted,

Electronic signature: /Jill Gorny Sloper, /
Jill Gorny Sloper, Esq.
Registration No.: 60,760
NELSON MULLINS RILEY & SCARBOROUGH LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 217-4630
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	15260888
Filing Date	2016-09-09
First Named Inventor	Leonard BELL
Art Unit	1644
Examiner Name	J. L. Rogers
Attorney Docket Number	AXJ-114BUSCN3

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15260888
	Filing Date	2016-09-09
	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN3

	1	HILL et al., Nitric oxide consumption and pulmonary hypertension in patients with paroxysmal; nocturnal hemoglobinuria, Blood 105:Abstract 1046 (2005);	
	2	HILL et al., Protection of erythrocytes from human complement-mediated lysis by membranetargeted; recombinant soluble CD59: a new approach to PNH therapy, Blood 107(5):2131-; 2137 (2006);	
	3	HILL et al., Sustained Control of Hemolysis and Symptoms and Reduced Transfusion; Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with; Eculizumab Therapy, Blood, 1 04:Abstract 2823 (2004);	
	4	HILL et al., Sustained response and long-term safety of eculizumab in paroxysmal nocturnal; hemogloinuria, Blood 106:2559-2565 (2005);	
	5	HILL, et al. "Effect of Eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria" N Engl J Med, 350(6), 2004, 552-559	
	6	HILL, et al. "Improvement in the symptoms of smooth muscle dystonia during eculizumab therapy in paroxysmal nocturnal; hemoglobinuria" Haematologica, 90(online), 2005, 111-113;	
	7	HILLMAN et al., Effect of the complement inhibitor eculizumab on thromboembolism in; I patients with paroxysmal nocturnal hemoglobinuria, Blood 110(12):4123-4128 (2007);	
	8	HILLMEN et al., Eculizumab, a C5 Complement-blocking Antibody, Abolishes Hemolysis and Renders Hemolytic Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) Transfusion Independent, 44th ASH Annual Meetinq (Abstract) Blood 100 (11) (Abstract No. 154) (2002).	
	9	HILLMEN et al., Eculizumab, a c5 complement-blocking antibody, controls hemolysis in paroxysmal nocturnal hemoglobinuria (pnh) with responses maintained over a prolonged period of therapy, Blood 102(11):1858 (2003) (Abstract)	
	10	HILLMEN et al., Effect of eculizumab on hemolysis and transfusion requirements in patients; with paroxysmal nocturnal hemoglobinuria, N. Engl. J. Med. 350:552-559 (2004);	
	11	HILLMEN, et al., "Eculizumab, a C5 complement-blocking antibody, controls haemolysis in; paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged; period of therapy," British Journal of Haematology, Vol. 125 (Suppl. 1): 1-72, Abstract 5; (2004).;	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15260888
	Filing Date	2016-09-09
	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN3

	12	HILLMEN, et al., "The terminal complement inhibitor eculizumab reduces thrombosis in patients with paroxysmal nocturnal hemoglobinuria," Blood 108(11):Abstract 123 (2006) XP002482084. 48th Annual Meeting of The American~ociety of Hematology. December 9-12,2006.	
	13	HILLMEN, et al., The complement inhibitor eculizumab in paroxysmal nocturnal; hemoglobinuria, N. Engl. J. Med. 355:1233-1243 (2006);	
	14	HILLMEN, 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, Vo1.121, no. Supplement 1, page 87, XP009144758 (2003). 43rd; Annual Scientific Meeting of the British Society for Haematology, April 7-9, 2003.;	
	15	INOUE et al., Molecular genetics of paroxysmal nocturnal hemoglobinuria, Int. J. Hematol.; 77(2):107-112(2003);	
	16	JASINSKI et al. "A novel mechanisms of complement-independent clearance of red cells deficient in glycosyl phosphatidylinositollinked; proteins," Blood, April 2004, 103(7),2827-2834;	
	17	KAPLAN "Eculizumab Alexion" Current Opinion in Invest. Drugs, 2002, 3(7),1017-1023	
	18	KATO et al. "Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes"; Blood Reviews, 21:37-47 (2007)	
	19	KINOSHITA, Molecular pathogenesis of Paroxysmal Nocturnal Haemoglobinuria	
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	Filing Date	2016-09-09
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	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN3

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Signature	/Jill Gorny Sloper/	Date (YYYY-MM-DD)	2016-10-21
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

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	1	THOMAS et al., Inhibition of complement activity by humanized anti-C5 antibody and single; chain Fv, Mol. Imm. 33 (17-18):1389-1401 (1996);	
	2	VAKEVA et al., Myocardial infarction and apoptosis after myocardial ischemia and; reperfusion, Circulation 97:2259-2267 (1998).	

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Application Number:	15260888
International Application Number:	
Confirmation Number:	2558
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Anita Costa
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Attorney Docket Number:	AXJ-114BUSCN3
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11	Non Patent Literature	Alexion_completes_enrollment_in_Ph_II_kidney_disease_trial.pdf	29992 693f575b2bd0395315cbe9dd1ae0f9924069058e	no	2
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(71) Applicants *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US). **THE UNIVERSITY OF WESTERN ONTARIO** [CA/CA]; 1151 Richmond Street North, Stevenson-Lawson Building, Room 328, London, Ontario N6A 5B8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROTHER, Russell, P.** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US). **WANG, Hao** [CA/CA]; 480 Sandybrook Drive, London, Ontario N5X 4H2 (CA). **ZHONG, Zhen** [CA/CA]; 60 Nathaniel Court, London, Ontario N5X 2N5 (CA).

(74) Agents: **WU, Xuqiong** et al.; Fish & Neave IP Group of, Ropes & Gray LLP, One International Place, Boston, MA 02110-2624 (US).

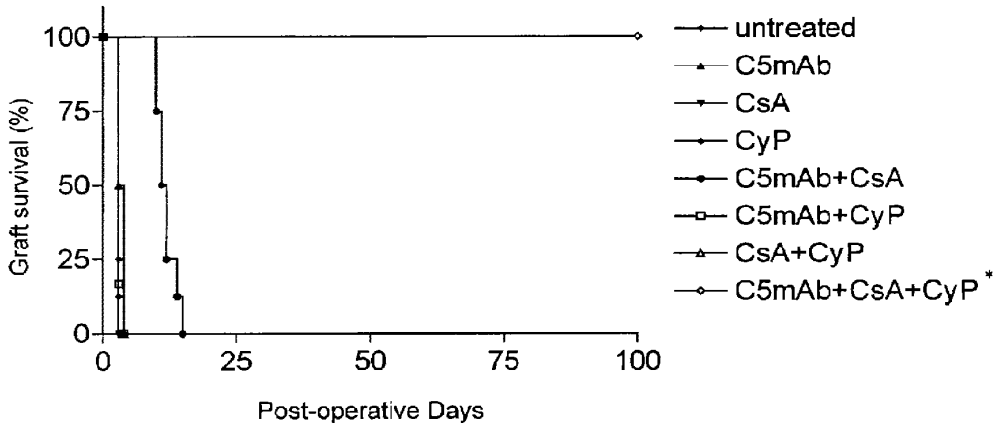
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(54) **Title:** PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY



(57) **Abstract:** Methods of prolonging survival of allotransplanted cells, tissues or organs are presented. These methods are directed to administering to the allotransplant recipient an inhibitor of complement activity together with one or more immunosuppressants. The inhibitor of complement activity is administered chronically. These methods have been determined to aid in preventing chronic rejection of allografts. These methods can additionally be used in cases in which the recipient has been presensitized to the allograft or in which there is an ABO mismatch between the allograft and the recipient.

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PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY

RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Patent Application No. 60/571,444, filed May 14, 2004, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to methods for prolonging survival of an allograft in a mammal. In particular, the present disclosure relates to prolonging survival of an allograft by administering an inhibitor of complement or terminal complement formation, especially an inhibitor of complement C5 cleavage, in addition to one or more drugs that are immunosuppressant.

BACKGROUND

Organ transplantation is the preferred treatment for most patients with chronic organ failure. Although transplantation of kidney, liver, lung, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle, it is limited by the medical/surgical suitability of potential recipients, an increasing shortage of donors, and premature failure of transplanted organ function.

Transplantation of cells, tissues and organs has become very common and is often a life-saving procedure. Organ transplantation is the preferred treatment for most patients with chronic organ failure. Despite great improvement in treatments to inhibit rejection, rejection continues to be the single largest impediment to successful organ transplantation. Rejection includes not only acute rejection but also chronic rejection. One-year survival rates for transplanted kidneys average 88.3% with kidneys from deceased donors and 94.4% with kidneys received from living donors. The corresponding five year survival rates for the transplanted kidneys are 63.3% and 76.5% (OPTN/SRTR Annual Report, 2002). For livers the one year survival rates are 80.2% and 76.5% for livers from deceased and living donors, respectively. The corresponding five year liver graft survival rates are 63.5% and 73.0% (OPTN/SRTR Annual Report, 2002). The use of immunosuppressant drugs, especially cyclosporin A and more recently tacrolimus, has dramatically improved the success rate of organ transplantation especially by preventing acute rejection. But as the numbers above

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show, there is still a need to improve the success rates, both short-term and especially long-term. As seen from the above numbers for kidney and liver transplants, the five year failure rates for these transplanted organs are on the order of 25-35%. In the year 2001 alone there were more than 23,000 patients who received an organ transplant of which approximately 19,000 were kidney or liver (OPTN/SRTR Annual Report, 2002). For this one year of transplants alone, with present techniques it can be expected that approximately 5,000-6,000 of these transplanted kidneys and livers will fail within 5 years. These numbers do not even include other transplanted organs or transplanted tissues or cells such as bone marrow.

There are multiple types of transplants. These are described in Abbas et al., 2000. A graft transplanted from one individual to the same individual is called an autologous graft or autograft. A graft transplanted between two genetically identical or syngeneic individual is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft or allograft. A graft transplanted between individuals of different species is called a xenogeneic graft or xenograft. The molecules that are recognized as foreign on allografts are called alloantigens and those on xenografts are called xenoantigens. The lymphocytes or antibodies that react with alloantigens or xenoantigens are described as being alloreactive or xenoreactive, respectively.

Currently more than 40,000 kidney, heart, lung, liver and pancreas transplants are performed in the United States each year (Abbas et al., 2000). Other possible transplants include, but are not limited to, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells. Unfortunately, there are many more candidates for a transplant than there are donors. To overcome this shortage, a major effort is being made to learn how to use xenografts. While progress is being made in this field, the fact is that at present most transplants are allografts. An allogeneic transplant, while presently being more likely to be successful than a xenogeneic transplant, must surmount numerous obstacles to be successful. There are several types of immunological attacks made by the recipient against the donor organ which can lead to rejection of the allograft. These include hyperacute rejection, acute vascular rejection (including accelerated humoral rejection and de novo acute humoral rejection), and chronic rejection. Rejection is normally a result of T-cell mediated or humoral antibody attack, but may include additional secondary factors such as the effects of complement and cytokines.

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An ever growing gap between the number of patients requiring organ transplantation and the number of donor organs available has become a major problem throughout the world. Park et al., 2003. Individuals who have developed anti-HLA antibodies are said to be immunized or sensitized. Gloor, 2005. HLA sensitization is the major barrier to optimal utilization of organs from living donors in clinical transplantation (Warren et al., 2004) due to the development of severe antibody-mediated rejection (ABMR). For example, more than 50% of all individuals awaiting kidney transplantation are presensitized patients (Glotz et al., 2002) who have elevated levels of broadly reactive alloantibodies, resulting from multiple transfusions, prior failed allografts, or pregnancy (Kupiec-Weglinski, 1996). The role of ABMR is currently one of the most dynamic areas of study in transplantation, due to recognition that this type of rejection can lead to either acute or chronic loss of allograft function. Mehra et al., 2003. Numerous cases of ABMR, including hyperacute rejection (HAR) or accelerated humoral rejection (ACHR), have been reported that are characterized by acute allograft injury that is resistant to potent anti-T cell therapy, the detection of circulating donor specific antibodies, and the deposition of complement components in the graft. ABMR with elevated circulating alloantibodies and complement activation that occurs in 20-30% of acute rejection cases has a poorer prognosis than cellular rejection. Mauiyyedi et al., 2002.

Highly presensitized patients, who exhibit high levels of alloantibodies, usually suffer an immediate and aggressive HAR. In clinical practice, with great efforts and significant advances in technology, HAR is avoided by obtaining a pretransplant lymphocytotoxic cross-match to identify sensitized patients with antibodies specific for donor HLA antigens. However, circulating antibodies against donor HLA or other non-MHC endothelial antigens may also be responsible for a delayed form of acute humoral rejection, which is associated with an increased incidence of graft loss. Collins et al., 1999. Therefore, development of a novel presensitized animal model to mimic ABMR in clinical settings would be beneficial to studies on the mechanism, and to the much needed progress in the management of allograft rejection in presensitized hosts.

Some highly presensitized patients can benefit from intervention programs such as immunoadsorption (Palmer et al.,1989; Ross et al., 1993; Kriaa et al., 1995), plasmapheresis and intravenous immunoglobulin (Sonnenday et al., 2002; Rocha et al., 2003), that have been designed and implemented to temporarily eliminate anti-donor antibodies. However, in addition to their benefits, the aforementioned therapies carry with them numerous drawbacks

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as some individuals are less susceptible to their effects (Kriaa et al., 1995; Hakim et al., 1990; Glotz et al., 1993; Tyan et al., 1994); they are extremely expensive, time-consuming, and risky (Salama et al., 2001). Moreover, the transient and variable effect of these protocols has limited their impact. Glotz et al., 2002; Kupin et al., 1991; Schweitzer et al., 2000. Therefore, developing novel strategies to reduce the risk and cost in prevention of ABMR would be beneficial to presensitized recipients receiving an allograft.

SUMMARY

Accordingly, methods of prolonging survival of transplanted cells, tissues or organs are provided. In particular, methods of prolonging survival of allotransplanted cells, tissues or organs are provided. These methods are directed to using one or more immunosuppressants in addition to an inhibitor of complement activity. Use of one or more immunosuppressants and an inhibitor of complement activity in the manufacture of one or more medicaments or medicament packages is also provided. Such medicaments or medicament packages are useful in prolonging survival of an allograft in a subject mammal.

In certain embodiments, the inhibition of complement activity is effected by chronic administration of a drug directed against complement C5. A preferred drug that inhibits complement activity is an antibody specific to one or more components of complement, for example, C5. In certain preferred embodiments, the antibody inhibits the cleavage of C5 and thereby inhibits the formation of both C5a and C5b-9. The antibody may be, e.g., a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), an antibody fragment (e.g., Fab), a single chain antibody, an Fv, or a domain antibody. The recipient is also treated with one or more immunosuppressive drugs, for example, cyclosporin A.

In certain embodiments, either an MHC mismatched recipient (i.e., a mammalian recipient of an MHC mismatched allograft), a presensitized recipient or an ABO mismatched recipient (i.e., a mammalian recipient of an AMB mismatched allograft) is treated. In this model, the recipient is again chronically treated with a complement inhibitor, preferably an anti-C5 monoclonal antibody, together with immunosuppressive drugs, preferably a chronic administration of cyclosporin A and a short-term administration of cyclophosphamide. This triple therapy results in extended graft survival in the presensitized allotransplant recipient.

The present disclosure also provides methods of prolonging survival of an allograft in a mammalian recipient by administering to the recipient agents that modulate the level and/or ratio of subclasses and/or isotypes of anti-donor immunoglobulins (Ig) in the recipient. In

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certain embodiments, an agent that reduces the level of anti-donor IgG1 in the recipient is preferred. In certain embodiments, an agent that increases the level of anti-donor IgG2a and/or IgG2b in the recipient is preferred. In certain embodiments, an agent that reduces the ratio of anti-donor IgG1/anti-donor IgG2a or IgG2b in the recipient is preferred.

The present disclosure also provides a method of prolonging survival of an allograft in a second mammalian recipient using an allograft that has been accommodated in a first mammalian recipient (i.e., the allograft has prolonged survival in the first recipient). The present disclosure further provides an allograft that is resistant to anti-donor antibodies in a mammalian recipient, and the allograft is prepared from a first recipient that has accommodated the allograft. In preferred embodiments, the first recipient has accommodated the allograft by receiving a treatment as described herein, such a triple therapy treatment involving administering to the first recipient a drug that inhibits complement activity and two immunosuppressive agents.

Further provided are pharmaceutical packages. A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation is stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized,

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chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show anti-donor antibody levels in presensitized versus unsensitized recipients under different treatments.

Figures 2A and 2B show comparison between triple therapy using anti-C5 antibody, CsA and CyP in presensitized allograft recipients and combination therapy using only anti-C5 antibody and CsA in presensitized allograft recipients. Figure 2A compares heart-allograft survival in various recipients under different treatments as indicated. Figure 2B shows histology and immunohistology, for example, for lymphocyte infiltration in heart allografts of recipients in different groups.

Figure 3 shows blocked terminal complement activity by anti-C5 antibody as compared to immunosuppressive agents.

Figures 4A-4D compare levels of anti-donor antibodies in presensitized recipients of allografts under monotherapy with anti-C5 antibody alone, double combination therapy with anti-C5 antibody and CsA, and triple combination therapy with anti-C5 antibody, CsA and CyP.

Figures 5A and 5B show change of ratios of IgG isotypes in allograft recipients that were untreated or under different treatments.

Figure 6 shows high-level expression of Bcl-2 and Bcl-xl proteins in long-term surviving heart grafts as compared to heart grafts of untreated animals.

Figure 7 shows improved second transplantation (re-transplantation) of an accommodated graft from a first transplantation recipient.

Figure 8 shows results from re-transplantation experiments.

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Figure 9 shows results from re-transplantation experiments.

DETAILED DESCRIPTION

Overview: Rejection of Transplants or Grafts

Hyperacute rejection occurs within minutes to hours after transplant and is due to preformed antibodies to the transplanted tissue antigens. It is characterized by hemorrhage and thrombotic occlusion of the graft vasculature. The binding of antibody to endothelium activates complement, and antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis and lead to vascular occlusion, the result being that the grafted organ suffers irreversible ischemic damage (Abbas et al., 2000). Hyperacute rejection is often mediated by preexisting IgM alloantibodies, e.g., those directed against the ABO blood group antigens expressed on red blood cells. This type of rejection, mediated by natural antibodies, is the main reason for rejection of xenotransplants. Hyperacute rejection due to natural IgM antibodies is no longer a major problem with allografts because allografts are usually selected to match the donor and recipient ABO type. Hyperacute rejection of an ABO matched allograft may still occur, usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well defined alloantigens expressed on vascular endothelial cells. Such antibodies may arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies (this prior exposure being referred to as "presensitization"). Abbas et al., 2000.

Acute rejection is a process of vascular and parenchymal injury mediated by T cells, macrophages, and antibodies that usually begins after the first week of transplantation. Abbas et al., 2001. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis. Both $CD4^+$ and $CD8^+$ cells may contribute to acute rejection. The destruction of allogeneic cells in a graft is highly specific and a hallmark of $CD8^+$ cytotoxic T lymphocyte killing. Abbas et al., 2000. $CD4^+$ T cells may be important in mediating acute graft rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in grafts, with some evidence available that indicates that $CD4^+$ T cells are sufficient to mediate acute rejection. Abbas et al., 2000. Antibodies can also mediate acute rejection after a graft recipient mounts a humoral immune

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response to vessel wall antigens and the antibodies that are produced bind to the vessel wall and activate complement. Abbas et al., 2000.

Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. Graft arterial occlusion may occur as a result of the proliferation of intimal smooth muscle cells (Abbas et al., 2000). This process is called accelerated or graft arteriosclerosis and can develop in any vascularized organ transplant within 6 months to a year after transplantation.

For a transplant to be successful, the several modes of rejection must be overcome. Multiple approaches are utilized in preventing rejection. This may require administration of immunosuppressants, often several types to prevent the various modes of attack, e.g., inhibition of T-cell attack, antibodies, and cytokine and complement effects. Prescreening of donors to match them with recipients is also a major factor in preventing rejection, especially in preventing hyperacute rejection. Immunoadsorption of anti-HLA antibodies prior to grafting may reduce hyperacute rejection. Prior to transplantation the recipient or host may be administered anti-T cell reagents, e.g., the monoclonal antibody OKT3, Anti-Thymocyte Globulin (ATG), cyclosporin A, or tacrolimus (FK 506). Additionally, glucocorticoids and/or azathioprine may be administered to the host prior to transplant. Drugs used to aid in preventing transplant rejection include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

Allografts are rejected in part by the activation of T cells. The transplant recipient mounts a rejection response following $CD4^+$ T cell recognition of foreign antigens in the allograft. These antigens are encoded by the major histocompatibility complex (MHC). There are both Class I and Class II MHC molecules. In humans the class I MHC molecules are HLA-A, HLA-B, and HLA-C. The class II MHC molecules in humans are called HLA-DR, HLA-DQ and HLA-DP. In mice the class I MHC molecules are H-2K, H-2D and H-2L and the class II MHC molecules are I-A and I-E. When $CD4^+$ T cells bind the foreign MHC antigens they are activated and undergo clonal proliferation. The activated T cells secrete cytokines which aid in activating monocytes/macrophages, B cells and cytotoxic $CD8^+$ T

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cells. The activated monocytes/macrophages release agents which result in tissue damage, the B cells produce alloantibodies which lead to complement mediated tissue destruction, and the $CD8^+$ T cells kill graft cells in an antigen-specific manner through induction of apoptosis and cell lysis.

Immunosuppressive Agents

The numerous drugs utilized to delay graft rejection (i.e., to prolong their survival) work in a variety of ways. Immunosuppressive agents are widely used. See Stepkowski, 2000, for a review of the mechanism of action of several immunosuppressive drugs. Cyclosporin A is one of the most widely used immunosuppressive drugs for inhibiting graft rejection. It is an inhibitor of interleukin-2 or IL-2 (it prevents mRNA transcription of interleukin-2). More directly, cyclosporin inhibits calcineurin activation that normally occurs upon T cell receptor stimulation. Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) enabling it to enter the nucleus and bind to interleukin-2 promoter. By blocking this process, cyclosporin A inhibits the activation of the $CD4^+$ T cells and the resulting cascade of events which would otherwise occur. Tacrolimus is another immunosuppressant that acts by inhibiting the production of interleukin-2.

Rapamycin (Sirolimus), SDZ RAD, and interleukin-2 receptor blockers are drugs that inhibit the action of interleukin-2 and therefore prevent the cascade of events described above.

Inhibitors of purine or pyrimidine biosynthesis are also used to inhibit graft rejection. These prevent DNA synthesis and thereby inhibit cell division including the ability of T cells to divide. The result is the inhibition of T cell activity by preventing the formation of new T cells. Inhibitors of purine synthesis include azathioprine, methotrexate, mycophenolate mofetil (MMF) and mizoribine (bredinin). Inhibitors of pyrimidine synthesis include brequinar sodium and leflunomide. Cyclophosphamide is an inhibitor of both purine and pyrimidine synthesis.

Yet another method for inhibiting T cell activation is to treat the recipient with antibodies to T cells. OKT3 is a murine monoclonal antibody against CD3 which is part of the T cell receptor. This antibody inhibits the T cell receptor and suppresses T cell activation.

Numerous other drugs and methods for delaying allotransplant rejection are known to and used by those of skill in the art. One approach has been to deplete T cells, e.g., by irradiation. This has often been used in bone marrow transplants, especially if there is a

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partial mismatch of major HLA. Administration to the recipient of an inhibitor (blocker) of the CD40 ligand-CD40 interaction and/or a blocker of the CD28-B7 interaction has been used (U.S. Patent 6,280,957). Published PCT patent application WO 01/37860 teaches the administration of an anti-CD3 monoclonal antibody and IL-5 to inhibit the Th1 immune response. Published PCT patent application WO 00/27421 teaches a method for prophylaxis or treatment of corneal transplant rejection by administering a tumor necrosis factor-α antagonist. Glotz et al. (2002) show that administration of intravenous immunoglobulins (IVIg) can induce a profound and sustained decrease in the titers of anti-HLA antibodies thereby allowing a transplant of an HLA-mismatched organ. Similar protocols have included plasma exchanges (Taube et al., 1984) or immunoadsorption techniques coupled to immunosuppressive agents (Hiesse et al., 1992) or a combination of these (Montgomery et al., 2000). Changelian et al. (2003) teach a model in which immunosuppression is caused by an oral inhibitor of Janus kinase 3 (JAK3) which is an enzyme necessary for the proper signaling of cytokine receptors which use the common gamma chain (γc) (Interleukins-2, -4, -7, -9, -15, -21), the result being an inhibition of T cell activation. Antisense nucleic acids against ICAM-1 have been used alone or in combination with a monoclonal antibody specific for leukocyte-function associated antigen 1 (LFA-1) in a study of heart allograft transplantation (Stepkowski, 2000). Similarly, an anti-ICAM-1 antibody has been used in combination with anti-LFA-1 antibody to treat heart allografts (Stepkowski, 2000). Antisense oligonucleotides have additionally been used in conjunction with cyclosporin in rat heart or kidney allograft models, resulting in a synergistic effect to prolong the survival of the grafts (Stepkowski, 2000). Chronic transplant rejection has been treated by administering an antagonist of TGF-β which is a cytokine involved in differentiation, proliferation and apoptosis (U.S. Patent Application Publication US 2003/0180301).

Complement and Transplant/Graft Rejection

The role of complement in transplant rejection is well known. This is especially true in the case of xenotransplantation, but complement also plays a role in allotransplant rejection. For review, see Platt and Saadi, 1999. One aspect of complement's role is that ischemia-reperfusion injury may occur at the time that an organ graft is reperfused with the blood of the recipient. Complement may also cause some manifestations of allograft rejection.

The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to

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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 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.

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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 (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

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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.

Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pretransplant crossmatch testing, this prototype of humoral rejection is now rarely observed (Regele et al., 2001). Data are now showing that humoral immune mechanisms might contribute to other types of allograft rejection (Regele et al., 2001). High levels of panel reactive antibodies indicating humoral presensitization were found to be associated with inferior kidney graft survival (Opelz, 1992), the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome (Jeannet et al., 1970; Halloran et al., 1992), and selective removal of recipient IgG by immunoadsorption reversed some rejection episodes indicating the contribution of humoral immune mechanisms to rejection (Persson et al., 1995; Böhmig et al., 2000). Complement activation within a graft might indicate antibody-mediated graft injury. The complement cleavage product C4d is a marker for activation of the antibody-dependent classical pathway. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome.

Recently increasing evidence indicates that complement activation significantly contributes to the sensitization of allograft recipients and the development of tissue injury in allografts (Platt et al., 1999). Antibodies are the most thoroughly investigated mediators of activating the classical complement pathway. Clinically, alloantibodies are known to activate complement (Baldwin et al., 2001). Halloran and Collins indicate that C4d deposition in peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies (Collins et al., 1999; Halloran, 2003). Further supporting evidence is seen in animals with complement inhibition (Pratt et al., 1996; Pruitt et al., 1991; Forbes et al., 1978) or deficiency (Pratt et al., 2000; Baurer et al., 1995) which exhibit significantly reduced inflammatory injury and lowered anti-donor immune responses. In ABMR, complement is suggested to be activated by the classical pathway and to play a key role in the pathogenesis (Collard et al., 1997). Although the role of complement in HAR or acute vascular rejection (AVR) following xenotransplantation has been well documented (Platt et al., 1999), precise mechanisms of complement in the pathogenesis of ABMR following allotransplantation has not yet been elucidated.

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The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition within the immune-mediated inflammatory response. As described above, C5a is a powerful anaphylatoxin and chemotactic factor. Cellular activation by C5a induces the release of multiple additional inflammatory mediators (Jose et al., 1983). The complement activation pathways (classical, alternative, or mannan-binding lectin pathway) ultimately lead to the formation of the cytolytic membrane attack complex C5b-9 (Kirschfunk, 2001), which can mediate both direct tissue injury by cell lysis, and proinflammatory cell activation at sublytic doses (Saadi et al., 1995; Papadimitriou et al., 1991). Therefore, blocking both C5a and C5b-9 generation may be required for the optimal inhibition of complement-mediated inflammatory response following transplantation. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b, preserving C3b-mediated opsonization of pathogenic microorganisms as well as solubilization and clearance of immune complexes (Liszewski, 1993).

The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction. In addition, complement inactivation by a functionally blocking anti-C5 monoclonal Ab (mAb) prevented HAR in xenotransplantation models (Kroshus et al., 1995; Wang et al., 1999).

Methods of delaying allotransplant rejection by administration of complement inhibitors have been tested. Published PCT patent application WO 92/10205 discloses the use of a combination of cyclosporin and a soluble complement receptor (sCR1) to inhibit rejection of a cardiac allotransplant in a presensitized rat model. Complement receptor 1 binds complements C3b and C4b. Soluble forms of complement receptor 1 occur naturally or can be generated via recombinant DNA procedures. These soluble complement receptors have inhibited in vitro the consequences of complement activation (U.S. Patent 6,057,131). In WO 92/10205, rats, which had been presensitized to the cardiac allograft they were receiving, were administered cyclosporin A intramuscularly at 10 mg/kg/day beginning two days prior to transplant and continued until the time of graft rejection. Additionally, soluble complement receptor 1 (sCR1) was administered as a single intravenous bolus at 15 mg/kg immediately prior to reperfusion of the graft. Control animals with no drug treatment had the

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graft rejected at an average of 3.8 days. Those administered cyclosporin A alone rejected the grafts at an average of 57 days (this was quite variable with two rats rejecting quickly at 2 and 4 days and a third rat rejecting at 166 days). Rats administered sCR1 alone rejected the grafts at an average of 44 days. Those rats administered the combination of cyclosporin A and sCR1 rejected the grafts at an average of 147 days. The combination of chronic cyclosporin A and single bolus sCR1 was seen to result in a synergistic effect greatly prolonging the time until graft rejection. Earlier studies by Pruitt and Bollinger (1991) used a similar model of a presensitized rat allograft to show that administration of sCR1 alone to inactivate complement resulted in increased time before graft rejection.

Sims et al. (U.S. Patent 5,135,916) suggest using inhibitors of complement, e.g., CD59 or antibodies against C7 or C9 to block the formation of the C5b-9 complex, to treat the vascular endothelium of organs and tissues to be transplanted. This would prevent the C5b-9 initiated cell necrosis. The C5b-9 inactivators would be added to the perfusate or storage medium to protect the vascular lining cells from ongoing complement activation during in vitro storage. Additionally the organ or tissue would be protected from the cytolytic and thrombotic effects arising from complement activation initiated upon transplantation, thereby circumventing complement mediated acute rejection. Sims et al. (U.S. Patent 5,573,940 and U.S. Patent 6,100,443) also teach a method of expressing CD59 in the transplanted tissue or organ to protect the transplanted organ from rejection. This can be accomplished by transfecting the cells being transplanted.

Although the several drugs developed to date in combination with methods of prescreening donors and recipients to match the donor allograft to the recipient have over time increased the average length of time of survival of allografts, many allografts are nonetheless rejected during the life-time of the recipient. In general, the prior art advances have mainly been directed to overcoming acute graft rejection. Further, the role of activated terminal complement components in antibody-mediated allograft rejection has not been examined using inhibitors that specifically target the complement cascade at the C5 protein level. The methods described herein and as exemplified in the Examples advance the allotransplant art by inhibiting chronic rejection of allografts, in particular, allografts in a presensitized recipient. New methods are presented for further prolonging allograft survival by using a proper combination of immunosuppressive drugs in combination with a chronic administration of a complement inhibitor.

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Methods and Uses

The methods disclosed herein are used to prolong allograft survival. The methods generally include administering an inhibitor of complement activity in combination with one or more immunosuppressants.

Suitable complement inhibitors are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C5a, C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). Proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and prevents it from being cleaved into C5a and C5b thereby preventing the formation not only of C5a but also the C5b-9 complex. Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

Suitable immunosuppressants include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

An allograft can include a transplanted organ, part of an organ, tissue or cell. These include, but are not limited to, heart, kidney, lung, pancreas, liver, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells.

At least part of the reason for the failure of allografts is that one response by the recipient of an allograft is the activation of complement. This results in the formation of C5a and C5b-9 which are potent proinflammatory molecules which aid in causing graft failure. Without wishing to be bound by any proposed theory, Applicants theorized that inhibiting the

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formation of C5a and C5b-9 or inhibiting C5a and C5b-9 which was present would aid in preventing graft failure. Furthermore, it was theorized that so long as the allograft is present, the recipient will continue to attempt to mount an immune response against the graft, and this response will include attempts to produce C5a and C5b-9. If not prevented, this complement response will lead to acute vascular rejection in the short term and could contribute to chronic graft rejection in the long term. Prior art methods of using inhibitors of complement activity were limited to administering these inhibitors only at the time of transplant. This was helpful in preventing acute rejection, but as the results disclosed herein illustrate, improved results are obtained by administration of such inhibitors for a longer term. This long-term administration aids in preventing a chronic rejection of the allograft as opposed to only aiding in preventing an acute rejection. The result is a longer term survival of the allograft as compared to either not administering an inhibitor of complement activity or administering such an inhibitor only at the time of transplant of the allograft. Although very commonly it is desirable that the allograft will survive for the remaining lifetime of the recipient, there are times when the allograft is needed only for a shorter length of time, e.g., a bridge organ to bridge the time until the recipient's own organ can recover on its own, at which time the allograft will no longer be needed. The length of time such a graft will be needed will vary, but will usually be longer than the time at which acute rejection would occur and may be long enough for chronic rejection to occur. This period of desired survival for a bridge graft may be several months, e.g., six months.

To prove that long-term inhibition of complement activity will prolong allograft survival, experiments were performed in which complement activation was inhibited in a chronic fashion and not merely at the time of transplant. Chronic treatment means treatment during an extended period up to the lifetime of the allograft. This can be daily treatment but is not limited to daily treatment. Chronic treatment will maintain an effective amount of the drug in the allograft recipient. For example, a preferred method is to include the anti-C5 monoclonal antibody eculizumab in the treatment. In studies of persons suffering from paroxysmal nocturnal hemoglobinuria (PNH), eculizumab has been administered at a dose of 900 mg/patient once every 12-14 days. This dosing has been found to completely and consistently block terminal complement activity and has greatly inhibited the symptoms of PNH (Hillmen et al., 2004). The administered dose is able to block the effects of complement for approximately two weeks before the eculizumab is inactivated or removed from the body. Therefore, a chronic treatment of eculizumab may be, e.g., the administration

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of 900 mg to the allograft recipient once every two weeks for the remaining life-time of the patient. Similarly, other drugs can be delivered chronically as needed, whether this is on a daily basis or another schedule is required to maintain an effective amount of the drug in the allograft recipient. Because it is well known that graft rejection can be caused by more than just complement activation, e.g., by T cell activity, the experiments included immunosuppressants such as cyclosporin to further aid in preventing graft rejection.

A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., 1987). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, a single-chain antibody, a domain antibody and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., 1986), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies when the recipient of the allograft is a human.

Administration and Formulations

Administration of the inhibitor of complement activity is performed according to methods known to those of skill in the art. These inhibitors are administered preferably before the time of allograft transplantation or at the time of transplantation with administration continuing in a chronic fashion. These inhibitors can additionally be administered during a rejection episode in the event such an episode does occur.

The present disclosure also provides uses of a drug that inhibits complement activity and an immunosuppressive agent in the manufacture of a medicament or medicament package. Such medicament or medicament package is useful in prolonging allograft survival in a recipient, in particular, chronic survival of the allograft. In preferred embodiments, the

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medicament or medicament package is formulated and prepared such that it is suitable for chronic administration to the recipient of the allograft, for example, stable formulations are employed. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for concurrent administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for sequential (in either order) administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft.

A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

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In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form. A formulated product of the present disclosure can be included within a container, typically, for example, a vial, cartridge, prefilled syringe or disposable pen. A doser such as the doser device described in U.S. Patent 6,302,855 may also be used, for example, with an injection system of the present disclosure.

A "stable" formulation is one in which the drug (e.g., an antibody) or agent therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. For example, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8 °C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

An isotonic reconstituted formulation is preferable in certain embodiments. By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

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A "lyoprotectant" is a molecule which, when combined with a drug (e.g., antibody) of interest, significantly prevents or reduces chemical and/or physical instability of the drug (e.g., antibody) upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the drug (e.g., antibody) in the presence of the lyoprotecting amount of the lyoprotectant, the drug (e.g., antibody) essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl parahen, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

Accordingly, a stable lyophilized antibody formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation

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can be reconstituted to generate a stable reconstituted formulation having an antibody concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the antibody concentration in the pre-lyophilized formulation. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, the reconstituted formulation can be stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8 °C. for at least about 30 days. See U.S. Patent 6,821,515. In certain embodiments, the reconstituted formulation is isotonic.

When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where a subject patient requires frequent administrations of the drug or antibody and/or agent to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

The present disclosure also provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of an antibody and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable.

An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a desirable antibody concentration in the reconstituted formulation. The article of manufacture may further comprise a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

An injection system of the present disclosure may employ a medication delivery pen as described in U.S. Patent 5,308,341. Medication delivery pens have been developed to facilitate the self-administration of medication. A medication of the present disclosure can be a drug that inhibits complement activity, for example an antibody specific to complement C5,

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and/or an immunosuppressive agent. One medication delivery pen includes a vial holder into which a vial of insulin or other medication may be received. The vial holder is an elongate generally tubular structure with proximal and distal ends. The distal end of the vial holder includes mounting means for engaging a double-ended needle cannula. The proximal end also includes mounting means for engaging a pen body which includes a driver and dose setting apparatus. A disposable medication containing vial for use with the prior art vial holder includes a distal end having a pierceable elastomeric septum that can be pierced by one end of a double-ended needle cannula. The proximal end of this vial includes a stopper slidably disposed in fluid tight engagement with the cylindrical wall of the vial. This medication delivery pen is used by inserting the vial of medication into the vial holder. A pen body then is connected to the proximal end of the vial holder. The pen body includes a dose setting apparatus for designating a dose of medication to be delivery by the pen and a driving apparatus for urging the stopper of the vial distally for a distance corresponding to the selected dose.

The user of the pen mounts a double-ended needle cannula to the distal end of the vial holder such that the proximal point of the needle cannula pierces the septum on the vial. The patient then selects a dose and operates the pen to urge the stopper distally to deliver the selected dose. The dose selecting apparatus returns to zero upon injection of the selected dose. The patient then removes and discards the needle cannula, and keeps the prior art medication delivery pen in a convenient location for the next required medication administration. The medication in the vial will become exhausted after several such administrations of medication. The patient then separates the vial holder from the pen body. The empty vial may then be removed and discarded. A new vial can be inserted into the vial holder, and the vial holder and pen body can be reassembled and used as explained above.

Accordingly, a medication delivery pen generally has a drive mechanism for accurate dosing and ease of use. A dosage mechanism such as a rotatable knob allows the user to accurately adjust the amount of medication that will be injected by the pen from a prepackaged vial of medication. To inject the dose of medication, the user inserts the needle under the skin and depresses the knob once as far as it will depress. The pen may be an entirely mechanical device or it may be combined with electronic circuitry to accurately set and/or indicate the dosage of medication that is injected into the user. See U.S. Patent 6,192,891.

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The present disclosure also presents controlled-release or extended-release formulations suitable for chronic and/or self-administration of a medication.

The various formulations can be administered to a patient in need of treatment (e.g., a recipient of an allograft) with the medication (e.g., an antibody of the present disclosure and at least one immunosuppressive agent) by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

In certain embodiments, a formulation is administered to the patient by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease® and Genject® devices); injector pens (such as the GenPen®); needleless devices (e.g. MediJector® and BioJector®); and subcutaneous patch delivery systems.

The present methods and uses are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. The following abbreviations are used herein: ABMR, antibody-mediated rejection; ACHR, accelerated humoral rejection; ACR, acute cellular rejection; AVR, acute vascular rejection; CsA, cyclosporin; CyP, cyclophosphamide; HAR, hyperacute rejection; MCP-1, monocyte chemotactic protein 1; MST, mean survival time; POD, postoperative day.

Example 1

Methods

Animals and Immunosuppressive Drugs Male adult C3H (H-2^k) mice and BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, Maine) weighing 25-30 g were chosen as donors and recipients, respectively. In the groups receiving immunosuppression, the recipients were injected with CsA (15 mg/kg/day, s.c., daily from day 0 to endpoint rejection or until day 100), or with CyP (40 mg/kg/day, i.v., on day 0 and 1), or with anti-C5 mAb (clone BB5.1, Alexion Pharmaceuticals Inc., 40 mg/kg/day, i.p., day 0–2, followed by twice a week, day 0-60). Animals were housed under conventional conditions at the Animal Care Facility,

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University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. Olfert et al., 1993.

Skin Presensitization Full-thickness skin grafts taken from C3H donors were cut into square pieces of 1×1 cm^2 and transplanted onto the back of the BALB/c recipients' thorax one week prior to heart transplantation from the same donors. Rejection was defined as complete necrosis of the skin grafts.

Abdominal and Cervical Cardiac Transplantation Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomen of presensitized BALB/c recipients by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava. In the groups with re-transplantation, second heart grafts harvested from either naive C3H mice or long-term surviving presensitized BALB/c recipients were transplanted into the cervical area of the recipients carrying a long-term surviving first abdominal heart graft by anastomosing the donor aorta and recipient carotid artery, and the donor pulmonary artery and recipient external jugular vein (end-to-side). The heart grafts were monitored daily until rejection unless otherwise indicated and rejection was defined as complete cessation of pulsation.

Experimental Groups Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: Group 1, mice with no treatment; Group 2, mice treated with CsA; Group 3, mice treated with CyP; Group 4, mice treated with CsA plus CyP; Group 5, mice treated with anti-C5 mAb; Group 6, mice treated with anti-C5 mAb plus CsA; Group 7, mice treated with anti-C5 mAb plus CyP; Group 8, mice treated with anti-C5 mAb in combination of CsA and CyP. When cardiac impulses were no longer palpable or at POD100, the grafts were removed for routine histology, immunohistochemistry and western blot analysis, serum samples were collected for flow cytometric analysis and complement hemolytic assay. Five additional animals were placed and sacrificed in groups 6 and 8 on POD3 (MST for groups 1-5, 7) to allow for comparisons at a uniform time point. Serum samples were also collected on POD 11, 21, 28 and 60 in Group 8 for detecting the sequential changes of anti-donor antibody levels and complement activity. In addition, when triple therapy treated presensitized recipients carried a first heart graft for 100 days, they were re-transplanted with a second heart. A naive C3H heart or a 100-day surviving C3H heart from another presensitized BALB/c recipient was used as the second heart. Eight animals were included in each re-transplant group.

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Graft Histology Tissue samples were fixed in 10% buffered formaldehyde. Specimens were then embedded in paraffin, and sectioned for H&E staining. The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist. Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage and lymphocyte infiltration. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change.

Immunohistochemistry Four micrometer sections were cut from tissue samples embedded in Tissue-Tek O.C.T gel (Optimum Cutting Temperature, Skura Finetek, Torrance, CA) mounted on gelatin-coated glass microscope slides and stained by a standard indirect avidin-biotin immunoperoxidase staining method using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Specimens were stained for $CD4^+$ and $CD8^+$ cells with biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.1.2, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and biotin-conjugated rat anti-mouse CD8 mAb (clone 53-6.7, Pharmingen, Franklin Lakes, NJ), respectively. Intragraft monocyte/macrophage infiltration was detected by staining with biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Mouse IgG and IgM deposition in grafts was detected using biotin-conjugated goat anti-mouse-IgG and goat anti-mouse-IgM (Cedarlane). For identification of complement deposition, sections were serially incubated with goat anti-C3 or anti-C5 polyclonal Abs (Quidel, San Diego, CA), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated-streptavidin (Zymed Laboratories, South San Francisco, CA). Slides were washed with phosphate-buffered saline between steps, and examined under light microscopy. Negative controls were performed by omitting the primary antibodies. The immunostaining was scored in five high-power fields of each section, and five independent experiments were performed. The sections of immunoperoxidase staining were graded from 0 to 4+ according to the staining intensity: 0, negative; 1+, equivocal; 2+, weak staining; 3+, moderate staining; and 4+, very intensive staining.

Flow Cytometry The circulating anti-donor specific IgG and IgM antibodies were evaluated in the recipient serum by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Glotz et al., (1993); Tyan et al. (1994). Briefly, C3H mouse splenocytes were isolated and incubated at 37 °C for 30 minutes with serum from naive control and experimental groups. To stain for total IgG, IgG1, IgG2a, IgG2b and IgM, the cells were washed and incubated with FITC-conjugated goat antibody specific for the Fc portion of mouse IgG or with

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phycoerythrin-conjugated goat antibody specific for the μ-chain of mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or with FITC-conjugated goat anti-mouse IgG1 (CALTAG Laboratories, Burlingame, CA), or with FITC-conjugated goat anti-mouse IgG2a (CALTAG), or with FITC-conjugated goat anti-mouse IgG2b (CALTAG). After 1 hour of staining at 4 °C, the cells were washed with PBS, resuspended at 5×10^6/mL, and analyzed by flow cytometry for mean channel fluorescence intensity, which represents the antibody-binding reactivity.

Complement Hemolytic Assay The purified anti-C5 mAb was serially diluted twofold (175-0.1 μg/ml) in GVB^{2+} buffer (gelatin Veronal-buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1.8 mM sodium barbital) and added in triplicate (50 μl/well) to a 96-well plate. BALB/c mouse serum was diluted to 40% v/v with GVB^{2+} buffer and added (50 μl/ml) to the rows of the same 96-well plate such that the final concentration of BALB/c mouse serum in each well was 20%. The plate was then incubated at room temperature for approximately 30 min while chicken erythrocytes were prepared. Chicken erythrocytes were washed 5×1 ml with GVB^{2+} buffer and resuspended to a final concentration of 5×10^7/ml in GVB^{2+}. Four milliliters of the chicken erythrocytes were sensitized by adding anti-chicken RBC polyclonal antibody (Intercell Technologies, Hopewell, NJ, 0.1% v/v) and the cells were incubated at 4 °C for 15 min with frequent vortexing. The cells were then washed 2×1 ml with GVB^{2+} and resuspended to a final volume of 2.4 ml in GVB^{2+}. The chicken erythrocytes (30 μl/well, 2.5×10^6 cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000×g for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at OD 415 nm using a microplate reader and the percentage of hemolysis was determined using this formula:

$$\%\ \text{hemolysis} = 100 \times \frac{(\text{OD sample}) - (\text{OD } GVB^{2+} \text{ control})}{(\text{OD 100\% lysed control}) - (\text{OD } GVB^{2+} \text{ control})}$$

with 100% lysed control obtained by the addition of 100 μl GVB^{2+} containing 0.1% NP-40 to the 30 μg/ml of chicken erythrocytes as prepared above.

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Western Blot Analysis Sonication of frozen heart samples was performed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) at 4 °C for 1 minute at 10-second intervals, followed by microcentrifugation at 13,000 rpm for 10 minutes at 4 °C. Clarified supernatants were immediately quantitated in triplicate for protein content using Detergent-compatible protein assay kit (BIO-RAD). Heart lysates (10μg protein/well) were separated on NuPAGE, 4-12% gradient Bis-Tris gels and MES buffer system (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size; Invitrogen) using a semi-dry transfer apparatus (BIO-RAD). Membranes were cut appropriately at the correct molecular weights to allow the development of the blots with two different primary antibodies per blot such that each blot was exposed to a test antibody and an internal control antibody to insure equal sample loading. The test primary antibodies including anti-Bcl-2 (N-19) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) and anti-Bcl-XS/L (M-125) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) were used to detect intragraft expression of Bcl-2 and Bcl-xl proteins. Anti-calsequestrin rabbit polyclonal sera (Calbiochem) were used as internal control primary antibody (Kobayashi et al., 1999). Detection of primary antibody binding was performed as previously described (Arp et al., 1996) by exposing washed incubated blots to a polyclonal goat anti-rabbit IgG fraction conjugated to horseradish peroxidase (HRP; Roche Laboratories) and then appropriately developed by exposure to enhance chemiluminescence for HRP-conjugated antibodies (Roche Laboratories).

Statistical Analysis The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the rank-log test. Histological and immunohistological findings were analyzed using the Mann-Whitney U test. Flow cytometric data and western blot data were analyzed using one-way ANOVA. Differences with p values less than 0.05 were considered significant.

Example 2

Presensitization with C3H donor skin graft induces antibody-mediated ACHR in heart allografts of BALB/c recipients.

To develop a suitable small animal model that mimics presensitized patients in the clinic and to study ABMR, a novel, fully MHC-mismatched mouse ABMR model has been developed through presensitization of mouse recipients. In this model, BALB/c recipients were presensitized with C3H donor skin grafts one week prior to heart transplantation from the same donor. Seven days after donor skin presensitization, serum level of anti-donor IgG,

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but not IgM antibody was markedly elevated and reached to a peak level in the presensitized BALB/c recipients (Figure 1A). Heart transplantation from same donor was then performed in these highly sensitized recipients. Without immunosuppression, C3H heart grafts were rapidly rejected in 3.1 ± 0.4 days by ACHR, characterized by severe thrombosis, hemorrhage and infarction (Figure 1B-a). In contrast, same heart grafts in unsensitized BALB/c recipients (with mean survival time, MST of 8.2 ± 0.8 days) show the normal histology on post-operative day (POD) 3 (Figure 1B-b). When compared to unsensitized BALB/c recipients at the same day, heart grafts in presensitized animals revealed massive IgG antibody and complement (C3 and C5) deposition, but minimal $CD4^+$ and $CD8^+$ cell infiltration (Table 1). Furthermore, circulating anti-donor IgG levels in presensitized recipients were significantly higher than those of unsensitized same recipients receiving a heart graft on POD3 ($P< 0.01$, Figure 1C). However, anti-donor IgM remained at very low levels both in circulation (Figure 1C) and in heart grafts (Table 1) and it showed no significant difference between unsensitized and presensitized recipients. In addition, normal levels of complement hemolytic activity were shown in both presensitized and unsensitized heart recipients without treatment (Figure 1D). These data indicate that this is an ideal transplant model to study ABMR in presensitized recipients in which complement plays an important role in the pathogenesis.

Table 1.

Comparison of immunohistological changes of C3H heart allografts in unsensitized and presensitized BALB/c recipients on POD3

Groups	Unsensitized	Presensitized
IgG	1+	4+
IgM	1+	1+
C3	2+	3+
C5	2+	3+
CD4	0	1+
CD8	0	1+

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Grades for immunoperoxidase staining: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb in combination with CsA and CyP prevents ABMR and achieves indefinite heart allograft survival in presensitized mouse recipients.

Complement has been shown to play an important role in ABMR. However, the inhibitory effect of functionally blocking terminal complement cascade at the C5 level in highly sensitized recipients is unknown. In the study presented herein, the presensitized model was used to study the efficacy of anti-C5 mAb either alone or combined with CsA and /or CyP in prevention of ABMR. As presented in Figure 2A, treatment with either CsA or CyP or the two drugs in combination did not prevent ABMR and grafts were rejected in 3.0 ± 0.0 days, 3.3 ± 0.5 days and 3.5 ± 0.6 days, respectively with typical pathological features of ACHR including intravascular thrombosis and interstitial hemorrhage (Figure 2B-b, c, d), which were indistinguishable from heart grafts in untreated presensitized BALB/c recipients (Figure 2B-a). Anti-C5 monotherapy or combined with CyP was not able to improve graft survival and heart grafts were rejected by ACHR (Figure 2B-e, f) in 3.5 ± 0.6 days and 3.2 ± 0.4 days, respectively (Figure 2A). Although the combination therapy of anti-C5 mAb and CsA, the protocol capable of inducing long-term heart allograft survival in unsensitized animals, marginally prolonged graft survival in this presensitized model, heart grafts were also rejected by severe humoral rejection with vasculitis, thrombosis, hemorrhage and minimal cell infiltration (Figure 2B-g) in 11.9 ± 1.8 days (Figure 2A). In contrast, triple therapy of anti-C5 mAb in combination of CsA and CyP achieved indefinite heart graft survival over 100 days (Figure 2A) in presensitized animals ($P<0.01$ vs. the animals without treatment or treated with either monotherapy or two drugs in combination) with no evidence of rejection (Figure 2B-h). In this presensitized mouse model, as shown in Table 2, only minor intragraft $CD4^+$ and $CD8^+$ cell infiltration was observed in the recipients that rejected their heart grafts within 3 days. However, the number of these T cells was slightly increased if heart grafts survived longer in anti-C5 mAb plus CsA-treated recipients at the time of rejection (POD11) and in triple therapy-treated recipients at early stages of graft survival (e.g. POD11). Furthermore, with continuous treatment of CsA in the triple therapy group, $CD4^+$ and $CD8^+$ cell infiltration was inhibited in long-term surviving heart grafts on POD60 and 100. In addition, moderate intragraft Mac-1^+ cell infiltration, including monocytes and macrophages, was found in untreated and CsA-, CyP- or CsA plus CyP-treated animals,

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while the infiltration of these cells was significantly reduced in anti-C5 mAb treated animals (Table 2). These results indicate that functionally blocking anti-C5 mAb enables the use and efficacy of conventional immunosuppressive agents, thereby preventing ABMR and achieving indefinite heart graft survival in presensitized recipients.

Table 2.

Grades for immunoperoxidase staining of heart allografts

in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Untreated	3	3+	3+	1+	1+	3+	4+	1+
CsA	3	3+	3+	1+	1+	3+	4+	1+
CyP	3	3+	3+	1+	1+	3+	3+	1+
CsA+CyP	3	3+	3+	1+	1+	3+	3+	1+
Anti-C5mAb	3	3+	0	1+	1+	2+	4+	1+
Anti-C5mAb+CsA	11	3+	0	2+	2+	2+	4+	1+
Anti-C5mAb+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	11	3+	0	2+	2+	1+	3+	1+
Anti-C5mAb+CsA+CyP	60	3+	0	1+	1+	0	2+	1+
Anti-C5mAb+CsA+CyP	100	3+	2+	0	0	0	2+	1+

Grades: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb completely inhibits total complement hemolytic activity and local C5 deposition in presensitized recipients receiving a heart allograft.

Anti-C5 mAb was previously shown to block the cleavage of complement protein C5 into the proinflammatory molecules C5a and C5b-9 (Kroshus et al., 1995), and to completely and consistently block terminal complement activity in mice (Wang et al., 1999). In the current study, terminal complement activity was measured by assessing the ability of

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recipient mouse sera to lyse antibody presensitized chicken erythrocytes and was compared at the same time-point (POD3). Treatment of mice with either CsA or CyP or the two drugs in combination had no effect on terminal complement activity, while treatment with anti-C5 mAb either alone or combined with CsA or/and CyP completely inhibited this activity (Figure 3; $P<0.01$, vs. naive and untreated animals, as well as CsA-, CyP-, or CsA plus CyP-treated animals). In addition, sera obtained from anti-C5 mAb treated animals at several earlier time points showed similarly diminished hemolytic activity, suggesting that serum terminal complement was inhibited throughout the treatment period. Furthermore, local C5 deposition in heart grafts was completely prevented in the anti-C5 mAb treated presensitized recipients, but not in untreated, or CsA-, CyP- and CsA plus CyP-treated presensitized animals (Table 2). As predicted, treatment with anti-C5 mAb did not prevent C3 deposition in the grafts (Table 2). These results suggest anti-C5 therapy completely blocks total complement activity after cardiac allografting in highly sensitized recipients.

Long-term surviving heart grafts in presensitized animals are resistant to humoral injury in the presence of low level of anti-donor antibodies and complement- a situation of accommodation.

To further investigate the role of anti-C5 mAb in humoral rejection, anti-donor alloantibody levels were measured in recipient sera by flow cytometry and intragraft antibody deposition by using immunostaining techniques in different groups. Figure 4A shows that on POD3 untreated presensitized BALB/c recipients had high levels of circulating anti-donor IgG antibodies. When presensitized recipients receiving either monotherapy or two drugs in combination, CsA and/or CyP partially down-regulated circulating anti-donor IgG levels, while treatment with anti-C5 mAb either alone or combined with CsA or CyP did not further affect anti-donor antibody levels at the same day. In contrast, with triple therapy of anti-C5 mAb, CsA and CyP, a high level of circulating anti-donor IgG was gradually downregulated and reached a low level on POD60, thereafter remaining at this level until day 100 (Figure 4B). Similar to levels of circulating antibodies in the different treatment groups, Table 2 shows that strong deposition of anti-mouse IgG was present in the rapidly rejected heart grafts of presensitized animals with no treatment or treated with monotherapy or two drugs in combination therapy. Interestingly, with triple therapy, IgG antibody deposition was gradually attenuated to a mild level in the long-term surviving heart grafts on POD 100 (Figure 4C-a, Table 2). In this model, IgM remained at very low levels in either circulation (Figure 4A, B) or transplanted heart grafts (Figure 4C-b, Table 2) in presensitized recipients

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with or without treatment. In addition, treatment with anti-C5 mAb eliminated complement activity to an undetectable level until day 60, followed by a progressive recovery to predepletion levels on POD 100 after discontinuation of anti-C5 therapy in presensitized mouse recipients receiving triple therapy (Figure 4D). Furthermore, intragraft C5 deposition was also detected in 100-day surviving presensitized animals (Table 2). These data demonstrate that ongoing transplant accommodation occurs in triple therapy treated presensitized recipients despite the presence of anti-graft antibodies and complement activation.

Anti-C5 mAb in combination with CsA and CyP reduces the IgG1/IgG2a ratio and leads to a shift in IgG subclass to IgG2b in recipients with accommodated grafts.

To determine whether anti-C5 mAb-based triple therapy would induce a shift in IgG subclass, which may be associated with accommodation, serum levels of anti-donor IgG subclasses of IgG1, IgG2a and IgG2b were compared between untreated recipients and the recipients with accommodated heart graft. Sera from untreated recipients contained predominant IgG1 isotype, indicated by a high ratio of IgG1/IgG2a (Figure 5A). In contrast, a significant reduction in the ratio of IgG1/IgG2a was observed in the recipients carrying accommodated grafts (Figure 5A, $P<0.01$). Furthermore, presensitized recipients with the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared to the same recipients with rejected grafts (Figure 5B, $P<0.01$). In addition, the pattern of IgG isotypes in the recipients treated with either monotherapy or two drugs in combination is indistinguishable from that of untreated animals. These data indicate that anti-donor IgG1 isotype may be associated with graft rejection, while production of anti-donor IgG2b subclass may function as a protective antibody and plays an important role in the induction of accommodation.

Anti-C5 mAb in combination with CsA and CyP induces intragraft Bcl-2 and Bcl-xl expression in highly sensitized mouse recipients.

To determine whether a causal relationship exists between intragraft expression of protective proteins and graft resistance to humoral injury in this model, western blot analysis was employed to detect proteins of interest in heart graft tissues from highly sensitized mouse recipients. Long-term surviving heart grafts were found to express high levels of Bcl-2 and Bcl-xl proteins on POD100, and these proteins were detected as early as 12 days after heart transplantation in highly sensitized recipients receiving anti-C5 mAb-based triple therapy

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(Figure 6). In contrast, there were no Bcl-2 and Bcl-xl proteins expressed on heart grafts of untreated animals (Figure 6) or animals treated with either monotherapy or two drugs in combination therapy. This result suggests that graft resistance to humoral injury in indefinite surviving animals is associated with the protection provided by Bcl-2 and Bcl-xl proteins in this presensitized model.

Presensitized recipients with an accommodating first heart graft accept a second accommodated heart graft but reject a second naive heart graft from the same donors.

The ability of accommodated grafts to resist rejection has not been tested directly under pathophysiological conditions where naive grafts undergo rejection following allotransplantation. In this model, to determine whether presensitized recipients with an accommodating first heart graft will accept a second accommodated graft but reject a second naive graft, we performed re-transplantation scenarios. After the accommodated C3H heart grafts have survived to the 100-day point, the time at which low levels of alloantibodies were detected (Figure 4B) and complement activity has returned to pretreatment levels (Figure 4D), in presensitized BALB/c recipient treated with anti-C5 mAb-based triple therapy, these recipients received a second heart graft. Specifically, either a naive (Figure 7A) or a 100-day accommodated C3H heart (Figure 7B) from another presensitized BALB/c recipient was transplanted into the neck of the presensitized recipients carrying an accommodating first C3H heart. These recipients rejected a second naive heart at 6.6 ± 1.1 days (Figure 8A) with severe AVR (Figure 8B-a) while the first heart continued to survive. In contrast, when the accommodated hearts that had been already surviving for 100 days in different presensitized mice were used as second grafts, these grafts were accepted by the presensitized recipients carrying an accommodating first heart graft (Figure 8A). There was no sign of rejection in those accommodated second heart grafts 90 days after second transplantation (Figure 8B-b). These data indicate that accommodated grafts become resistant to the effects of anti-donor antibodies and complement that normally mediate allograft rejection in these presensitized recipients. Furthermore, the fact that the host of the accommodated graft rejected a new graft suggests that accommodation involves changes to the graft.

Presensitized recipients being treated with CsA reject accommodated heart grafts.

Another re-transplantation was performed to determine whether accommodation in this presensitized model would be caused by the changes in the grafts and/or the recipients. Specifically, after C3H heart grafts have been accommodated in presensitized BALB/c mice

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for 100 days, the accommodated heart graft will then be re-transplanted into a second presensitized BALB/c recipient being treated with CsA alone (Figure 7C), a therapy that can prevent cellular rejection but cannot prevent accelerated humoral rejection of a fresh C3H heart in presensitized recipients. The accommodated C3H heart grafts were rapidly rejected in CsA treated presensitized BALB/c recipients. After re-transplantation, the pathology in accommodated heart grafts was changed from normal (Figure 9A) to severe ACHR with massive interstitial hemorrhage but few cell infiltrates (Figure 9B). In addition, high levels of anti-donor IgG and normal levels of complement hemolytic activity in these recipients receiving an accommodated C3H heart were similar to those of CsA treated presensitized recipients receiving a naive C3H heart. This result further indicates that accommodation induced by anti-C5 mAb-based triple therapy can originate from mechanisms involving changes not only to the graft, but also to the recipient.

Example 3

Acute Vascular Rejection in a Heart Transplantation Model

Experiments were performed to determine whether inclusion of an inhibitor of formation of terminal complement would attenuate acute vascular rejection and whether the use of such an inhibitor in conjunction with an immunosuppressant would achieve long-term allograft survival. In this set of experiments an anti-C5 monoclonal antibody was used in conjunction with cyclosporin. The model used was an allograft heterotopic heart transplant from C3H mice into BALB/c mice. This model is a stringent acute vascular rejection model with the C3H and BALB/c mice being strongly MHC mismatched. The transplantations and other methods were performed as described in Wang et al. (2003).

Heterotopic Cardiac Transplantation

Intra-abdominal heterotopic cardiac transplantation was performed as previously described by Wang et al. (2003). Briefly, a median sternotomy was performed on the donor, and the heart graft was slowly perfused in situ with 1.0 ml of cold heparinized Ringer's lactate solution through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were transected, and the graft was removed from the donor. The graft was then revascularized with end-to-side anastomoses between the donor's pulmonary artery and the recipient's inferior vena cava as well as the donor's aorta and the recipient's abdominal aorta using 11-0 nylon suture. The beating of the grafted heart was monitored daily by direct abdominal palpation.

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The degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology. In certain instances, mice in which the graft was still functioning were sacrificed to perform histology.

Results

Mice (male 8-12 week old mice weighing 25 – 30 g) were split into six experimental groups with six to eight mice per group. Transplant occurred on day 0. Histological changes were checked at the endpoint (the endpoint being graft failure) or in some cases a mouse was sacrificed prior to graft failure. The dosage of BB5.1 which was administered (40 mg/kg body weight three times per week) was known from prior studies to completely inhibit terminal complement activity.

Group 1' (control) - mice were administered 0.75 mL of saline intraperitoneally on days -1, 0, 1 and 2. Subsequently these mice were treated with 0.75 mL of saline intraperitoneally three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 2' (cyclosporin A alone) - mice were administered 15 mg/kg body weight of cyclosporin A subcutaneously on a daily basis beginning at day 0 (day of transplant) until the endpoint.

Group 3' (anti-complement antibody alone) - mice were administered the anti-mouse C5 antibody BB5.1 (Frei et al., 1987) at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 4' (anti-complement antibody until day 14 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intravenously on days -1 through day 14 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint. Note that this differs from the other groups in that the BB5.1 was administered intravenously and on a daily basis.

Group 5' (anti-complement antibody until day 28 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until day 28 and were also administered

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cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint.

Group 6' (anti-complement antibody chronically until 100 days plus cyclosporin) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until 100 days and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until 100 days.

The results of this experiment are shown in Tables 3 and 4. Table 3 shows the survival time for the grafts. Table 4 sets forth the histological scores.

Table 3

Allograft Survival

Group (Treatment)	Individual Survival (days)	Mean Survival Time (days)
1'. Saline	8, 8, 8, 8, 8, 9	8.3 ± 0.5
2'. Cyclosporin A	14, 15, 15, 16, 16, 16, 17	15.5 ± 1.1
3'. BB5.1	7, 8, 8, 8, 8, 9	8.0 ± 0.6
4'. BB5.1 until day 14 + cyclosporin A	35, 38, 43, 45, 46, 47	42.3 ± 4.8
5'. BB5.1 until day 28 + cyclosporin A	77, 80, 80, 81, 82	80 ±1.9
6'. BB5.1 until day 100 + cyclosporin A	>100 days (7 mice; one sacrificed for histology)	> 100 days

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Table 4

Median Scores of Histological Changes of Heart Allografts at Necropsy

Groups	Vasc*	Infarc	Lymph	Throm	Hemo	Fibrin	PMN
1'. Saline (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
2'. Cyclosporin A (endpoint)	2.0	1.0	2.0	3.0	2.0	2.0	3.0
3'. BB5.1 (endpoint)	2.0	1.0	2.0	2.0	1.0	0.0	0.0
4'. BB5.1 until day 14 + cyclosporin A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5'. BB5.1 28 days + Cyclosporin A (post-operative day 8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5'. BB5.1 28 days + Cyclosporin A (endpoint)	0.0	0.0	1.0	1.0	2.0	1.0	0.0
6'. BB5.1 until day 100 + Cyclosporin A (post-operative day 100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available.

*Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate

The results indicate the synergistic effects of using a complement inhibiting drug in addition to an immunosuppressant. In untreated mice the grafts were rejected in about 8 days. Use of the immunosuppressant cyclosporin A alone on a daily, chronic basis resulted

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in an increase in graft survival until approximately 15 days post-transplant. The use of the anti-C5 antibody BB5.1 to inhibit formation of terminal complement had no effect on its own, graft rejection occurring at 8 days post-transplant as in the control group (Group 1'). The combination of BB5.1 through day 28 post-transplant plus cyclosporin A showed a synergistic effect with graft survival being extended until approximately day 80. A more surprising result is that of Group 5' in which BB5.1 and cyclosporin A were each administered chronically post-transplant. In this case the graft survival was for more than 100 days (as much data as presently available). Additionally, the histological results shown in Table 4 indicate that the administration of both BB5.1 and cyclosporin A protected the graft from changes much better than either BB5.1 or cyclosporin A alone, and that the chronic administration of BB5.1 and cyclosporin A protected the graft to such an extent that even at 100 days post-transplant there were no histological changes seen in the engrafted hearts. A survival time of 100 days in these models is considered to be the gold standard. A survival of 100 days in the model is believed to indicate that there will be an indefinite survival of the allograft. When BB5.1 administration was stopped after 28 days, the grafts were protected but they did begin to show some minimal to mild histological changes by about day 80 which was the time at which graft failure occurred.

The Group 4' mice were treated differently in that they were administered BB5.1 on a daily basis by an intravenous administration. These animals became ill, showing weight loss and urine retention and were sacrificed at a time at which the grafted hearts were still beating although their function had declined. This was the first group of mice studied and it is unknown why these ill effects were seen. These ill effects were not seen when the BB5.1 was administered intraperitoneally with a schedule of three times per week. As seen below in Example 4, daily administration of BB5.1 via an intraperitoneal route did not cause ill effects. Also, intravenous administration was not necessarily the cause of the illness in these animals. Intravenous administration of eculizumab (a human equivalent antibody to BB5.1 in that it binds to human C5) has been successfully administered intravenously without ill effects to humans in a study of PNH (Hillmen et al., 2004). Complement inhibitors may be administered by other routes in addition to intravenous and intraperitoneal, with all such routes being well known by those skilled in the art.

Example 4

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Accelerated Rejection in a Presensitized Heart Transplantation Model

A second set of experiments similar to those of Example 3 was performed but the recipient mouse was presensitized to the donor organ. In these experiments, the presensitization was brought about by prior transplantation of a skin graft. In general, presensitization can occur not only as a result of having received an earlier allograft, but can also be caused by having received multiple blood transfusions or in women who have been pregnant. Besides such presensitization methods, allografts with an ABO mismatch will be rapidly attacked and rejected because of preformed antibodies to the ABO antigens unless steps are taken to prevent such an attack.

Some mice in these studies were administered cyclophosphamide in addition to BB5.1 and/or cyclosporin A. For these experiments BALB/c recipient mice were presensitized with C3H skin grafts one week prior to heart transplantation from the same donor (using the method of Pruitt and Bollinger, 1991). This model is designed to mimic presensitized transplantation in humans, especially in relation to accelerated humoral rejection. Recipient mice were split into eight groups of six to eight mice each. The treatments were as follow.

Group 1" (control) - mice (male 8-12 week old mice weighing 25 – 30 g) were administered 0.75 mL saline intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint (graft rejection).

Group 2" (cyclosporin A alone) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight beginning on day 0 (day of transplant) until the endpoint.

Group 3" (BB5.1 alone) - mice were administered the anti-mouse complement monoclonal antibody BB5.1 at a dose of 40 mg/kg body weight delivered intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint.

Group 4" (cyclophosphamide alone) - mice were administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 5" (BB5.1 plus cyclosporin A) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint.

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Group 6" (BB5.1 plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 7" (cyclosporin A plus cyclophosphamide) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 8" (BB5.1 plus cyclosporin A plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until 100 days. These mice were also administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until 100 days. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1. Two mice in this group were sacrificed at day 60 for histological studies (no rejection had yet occurred) and the four remaining mice still had not rejected their grafts by day 100.

Additionally a control group of mice which was not presensitized and received only the saline treatment as for Group 1" was tested.

The results of these experiments are shown in Tables 5 and 6. Table 5 lists survival times for the grafts and Table 6 summarizes the histological results.

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Table 5

Allograft Survival

Groups (Treatment)	Individual survival (days)	Mean survival time (days)
No presensitization	8, 8, 8, 8, 8, 9	8.3 ± 0.5*
1". One skin presensitization	3, 3, 3, 3, 3, 3, 3, 4	3.1 ± 0.4
2". Cyclosporin A	3, 3, 3, 3	3.0 ± 0.0
3". BB5.1	3, 3, 4, 4	3.5 ± 0.6
4". Cyclophosphamide	3, 3, 3, 4	3.3 ± 0.5
5". BB5.1 + Cyclosporin A	10, 10, 11, 11, 12, 12, 14, 15	11.9 ± 1.8**
6". BB5.1 + Cyclophosphamide	3, 3, 3, 3, 3, 4	3.2 ± 0.4
7". Cyclosporin A + Cyclophosphamide	3, 3, 3, 4, 4, 4	3.5 ± 0.6
8". BB5.1 + Cyclosporin A + Cyclophosphamide	> 100 days (4 mice)	> 100***

*P < 0.01 group 1" vs. no presensitization

**P < 0.01 group 5" vs. groups 1"-4" and 6"-7".

***P<0.01 group 8" vs. groups 1"-7".

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Table 6

Median Scores of Histological Changes of Heart Allografts at Necropsy

Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
No presensitization (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
1". One skin presensitization (endpoint)	0.0	4.0	1.0	4.0	3.0	0.0	0.0
2". Cyclosporin A (endpoint)	0.0	4.0	0.0	3.0	3.0	N/A	N/A
3". BB5.1 (endpoint)	2.0	2.0	2.0	2.0	3.0	N/A	N/A
4". Cyclophosphamide (endpoint)	2.0	4.0	0.0	3.0	2.0	N/A	N/A
5". BB5.1 + Cyclosporin A (post-operative day 3)	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5". BB5.1 + Cyclosporin A (endpoint)	2.0	2.0	2.0	2.0	2.0	0.0	0.0
6". BB5.1 + Cyclophosphamide (endpoint)	1.0	3.0	0.0	3.0	2.0	N/A	N/A
7". Cyclosporin A + Cyclophosphamide (endpoint)	0.0	1.0	1.0	2.0	3.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 3)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8. BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 12)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-	0.0	0.0	1.0	0.0	0.0	N/A	N/A

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operative day 60)							
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 100)	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available.

*Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate

The results shown in Table 5 indicate a difference between the presensitized mouse model and the nonpresensitized mouse model as used in Example 3. The results indicate that in the absence of presensitization, grafts are rejected in approximately 8 days in the absence of treatment with any drugs. Presensitizing the animals causes a more rapid rejection, the rejection of the graft in the presensitized animals being in approximately 3 days in the absence of any drug treatment. Treatment with either BB5.1, cyclosporin A or cyclophosphamide had no effect upon graft survival, with the grafts being rejected in approximately 3-4 days in each of these groups of animals. The combination of BB5.1 and cyclosporin A showed some effect with rejection occurring about day 12. The combination of BB5.1 and cyclophosphamide had no protective effect with rejection occurring about day 3. Similarly the combination of cyclosporin A and cyclophosphamide had essentially no protective effect with rejection occurring at 3-4 days. Very surprisingly, the combination of all three drugs (chronic administration of BB5.1 and cyclosporin plus administration of cyclophosphamide at the time of transplant) showed a highly synergistic effect with all of the mice surviving for more than 100 days. Again, a survival of 100 days in this model is considered to be the gold standard and assumes an indefinite survival.

These results as well as the histological results as shown in Table 6 indicate that the combination of chronic treatment with a complement inhibitor and an immunosuppressant such as cyclosporin A in treating a presensitized mouse results in some attenuation of accelerated rejection. Treatment of these animals additionally with cyclophosphamide at the time of transplant and on the first day after transplant results in a much greater time of survival, no rejection having been seen by at least day 100.

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It will be appreciated that the methods and compositions of the instant disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the disclosure. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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LIST OF REFERENCES

The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are incorporated herein by reference in their entirety, and for convenience, are referenced by author and date in the text and respectively grouped in the following List of References.

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The contents of all references cited herein are incorporated by reference in their entirety.

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CLAIMS

1. A method to prolong survival of an MHC mismatched allograft in a recipient mammal, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

2. The method of claim 1 wherein said mammal is a human.

3. The method of claim 2 wherein said MHC mismatched allograft is an HLA mismatched allograft.

4. The method of claim 1 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

5. The method of claim 4 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

6. The method of claim 5 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

7. The method of claim 5 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

8. The method of claim 5 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

9. The method of claim 5 wherein said antibody fragment is pexelizumab.

10. The method of claim 5 wherein said whole antibody is eculizumab.

11. The method claim 10 wherein said eculizumab is administered once every 2 weeks.

12. The method of claim 1 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

13. The method of claim 1 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

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14. The method of claim 1 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

15. The method of claim 1 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

16. The method of claim 1 wherein more than one immunosuppressive drug is administered.

17. The method of claim 1 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

18. The method of claim 17 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

19. The method of claim 1 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

20. The method of claim 1 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

21. The method of claim 1 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

22. The method of claim 1 wherein said allograft survives for the remaining life-time of said mammal.

23. The method of claim 2 wherein said allograft survives for at least six months.

24. The method of claim 2 wherein said allograft survives for at least one year.

25. The method of claim 2 wherein said allograft survives for at least five years.

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26. The method of claim 2 wherein said allograft survives for the remaining life-time of said human.

27. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

28. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

29. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

30. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

31. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

32. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

33. The method of claim 1 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

34. The method of claim 1 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

35. The method of claim 34 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

36. A method to prolong survival of an allograft in a recipient mammal presensitized to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

37. The method of claim 36 wherein said mammal is a human.

38. The method of claim 36 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

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39. The method of claim 38 wherein said drug that inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

40. The method of claim 39 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

41. The method of claim 39 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

42. The method of claim 39 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

43. The method of claim 39 wherein said antibody fragment is pexelizumab.

44. The method of claim 39 wherein said whole antibody is eculizumab.

45. The method claim 44 wherein said eculizumab is administered once every 2 weeks.

46. The method of claim 36 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

47. The method of claim 36 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

48. The method of claim 36 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

49. The method of claim 36 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

50. The method of claim 36 wherein more than one immunosuppressive drug is administered.

51. The method of claim 36 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

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52. The method of claim 51 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

53. The method of claim 51 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

54. The method of claim 36 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

55. The method of claim 36 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

56. The method of claim 36 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

57. The method of claim 36 wherein said allograft survives for the remaining life-time of said mammal.

58. The method of claim 37 wherein said allograft survives for at least six months.

59. The method of claim 37 wherein said allograft survives for at least one year.

60. The method of claim 37 wherein said allograft survives for at least five years.

61. The method of claim 37 wherein said allograft survives for the remaining life-time of said human.

62. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

63. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

64. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

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65. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

66. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

67. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

68. The method of claim 36 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

69. The method of claim 36 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

70. The method of claim 69 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

71. A method to prolong survival of an allograft in a recipient mammal which is an ABO mismatch to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

72. The method of claim 71 wherein said mammal is a human.

73. The method of claim 71 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

74. The method of claim 73 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

75. The method of claim 74 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

76. The method of claim 74 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

77. The method of claim 74 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

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78. The method of claim 74 wherein said antibody fragment is pexelizumab.

79. The method of claim 74 wherein said whole antibody is eculizumab.

80. The method claim 79 wherein said eculizumab is administered once every 2 weeks.

81. The method of claim 71 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

82. The method of claim 71 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

83. The method of claim 71 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

84. The method of claim 71 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

85. The method of claim 71 wherein more than one immunosuppressive drug is administered.

86. The method of claim 71 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

87. The method of claim 86 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

88. The method of claim 86 wherein said drug that inhibits complement activity is an antibody that inhibits cleavage of complement C5.

89. The method of claim 71 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

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90. The method of claim 71 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

91. The method of claim 71 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

92. The method of claim 71 wherein said allograft survives for the remaining life-time of said mammal.

93. The method of claim 72 wherein said allograft survives for at least six months.

94. The method of claim 72 wherein said allograft survives for at least one year.

95. The method of claim 72 wherein said allograft survives for at least five years.

96. The method of claim 72 wherein said allograft survives for the remaining life-time of said human.

97. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

98. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

99. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

100. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

101. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

102. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

103. The method of claim 71 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

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104. The method of claim 71 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

105. The method of claim 104 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

106. Use of a drug that inhibits complement activity and an immunosuppressive drug in the manufacture of a medicament or medicament package for prolong survival of an allograft in a mammal.

107. The use of claim 106, wherein more than one immunosuppressive drug is included in said medicament or medicament package.

108. The use of claim 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation suitable for concurrent administration to said mammal.

109. The use of claim 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation or formulations suitable for sequential administration to said mammal.

110. The use of claim 106, wherein said drug that inhibits complement activity is in a formulation suitable for chronic administration to said mammal.

111. The use of claim 106, wherein said immunosuppressive drug is in a formulation suitable for chronic administration to said mammal.

112. A method to prolong survival of allograft in a recipient mammal, comprising preparing an allograft from a first allograft accommodated by a first recipient mammal, wherein said first recipient mammal, after receiving said first allograft, has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

113. An allograft that has prolonged survival in a recipient mammal, wherein said allograft is prepared from a first recipient mammal and was transplanted to the first recipient mammal that has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

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114. A pharmaceutical package comprising a drug that inhibits complement activity and at least one immunosuppressive agent, wherein said drug and said agent are formulated for chronic administration.

115. The pharmaceutical package of claim 114, wherein said drug is an antibody in a lyophilized formulation comprising the antibody and a lyoprotectant.

116. The pharmaceutical package of claim 114, wherein said agent is in a lyophilized formulation comprising the agent and a lyoprotectant.

117. The pharmaceutical package of claim 114, wherein said drug and said agent are in the same lyophilized formulation comprising said drug, said agent, and a lyoprotectant.

118. The pharmaceutical package of claim 114, wherein said drug is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug in a formulation suitable for injection.

119. The pharmaceutical package of claim 114, wherein said agent is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said agent in a formulation suitable for injection.

120. The pharmaceutical package of claim 114, wherein said drug and said agent are in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug and said agent in a formulation suitable for injection.

121. The pharmaceutical package of claim 114, wherein said drug is present in unit dosage form.

122. The pharmaceutical package of claim 114, wherein said agent is present in unit dosage form.

123. A lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant, wherein said antibody formulation is suitable for chronic administration.

124. The lyophilized antibody formulation of claim 123, wherein the antibody inhibits the formation of terminal complement or C5a.

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125. The lyophilized antibody formulation of claim 123, wherein the antibody is a whole antibody or an antibody fragment.

126. The lyophilized antibody formulation of claim 125, wherein the antibody wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

127. The lyophilized antibody formulation of claim 125, wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

128. The lyophilized antibody formulation of claim 125, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

129. The lyophilized antibody formulation of claim 125, wherein said antibody fragment is pexelizumab.

130. The lyophilized antibody formulation of claim 125, wherein said whole antibody is eculizumab.

131. An injection system comprising a syringe, wherein said syringe comprises a cartridge containing an antibody that inhibits complement activity and is formulated suitable for injection.

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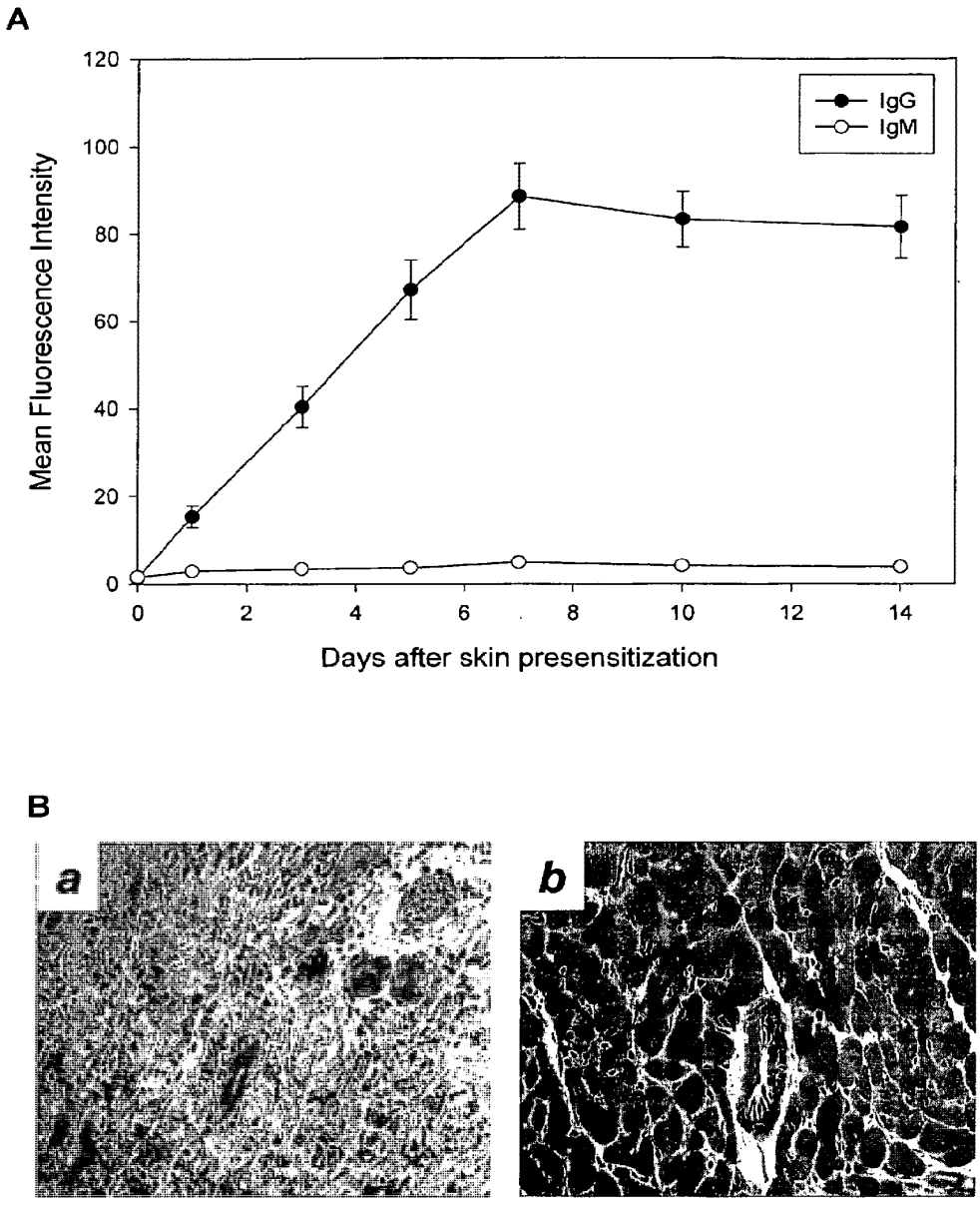


Figure 1A, B

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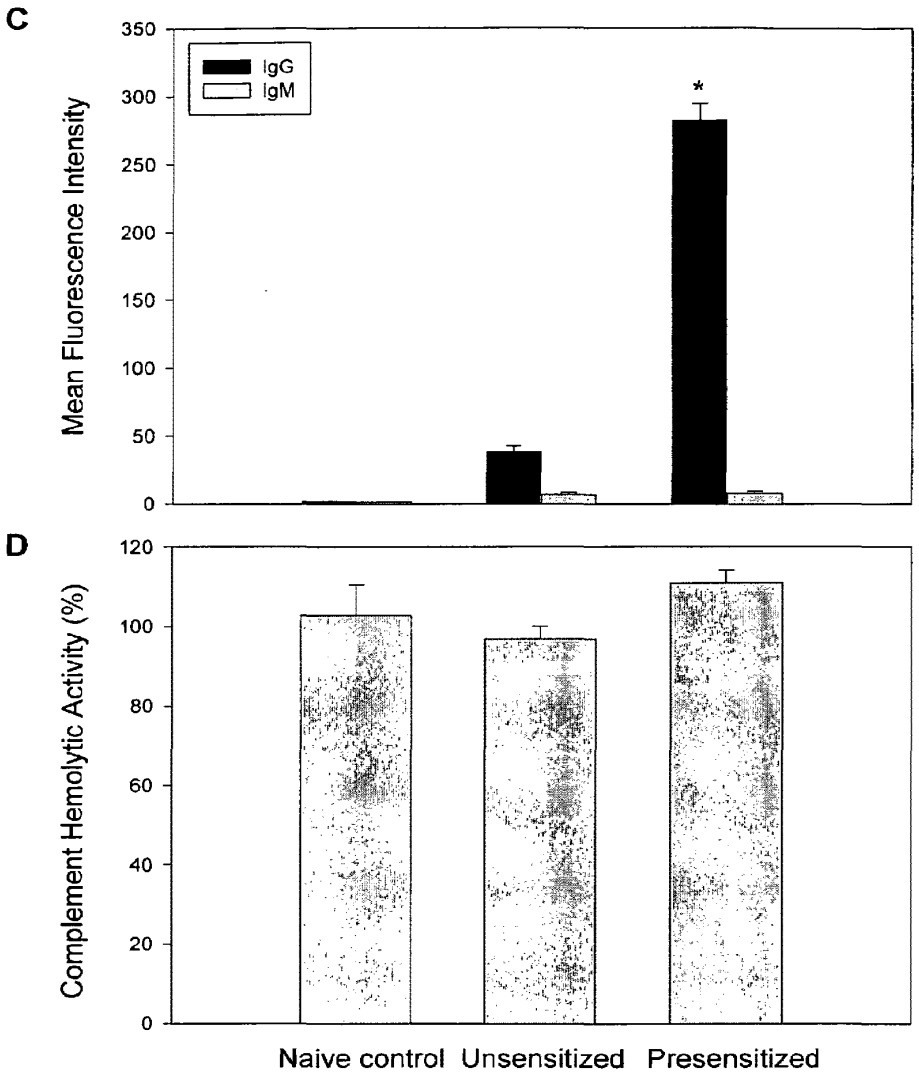


Figure 1C, D

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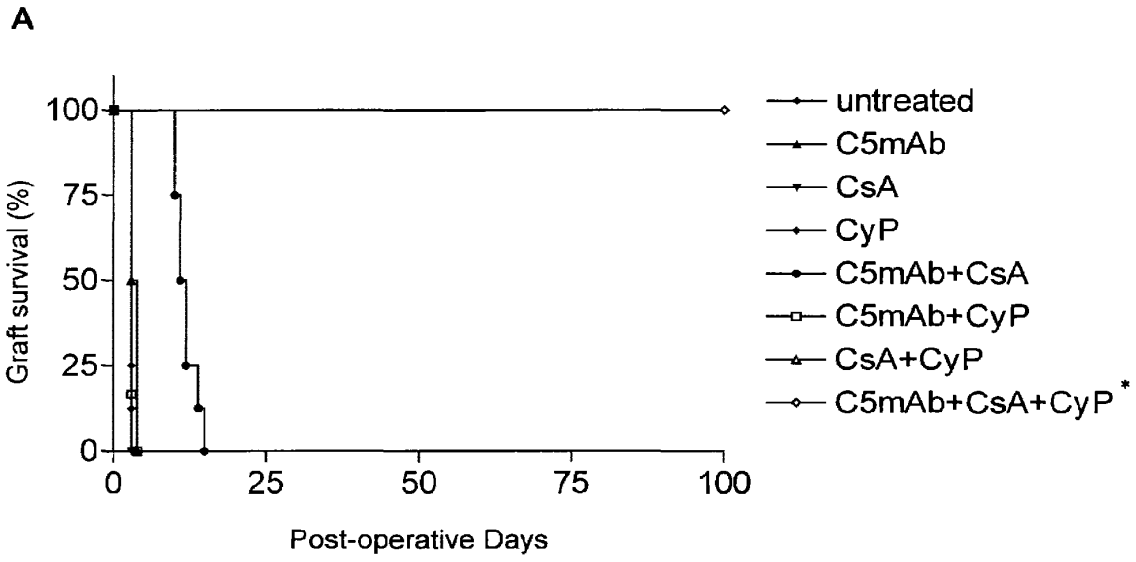


Figure 2A

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B

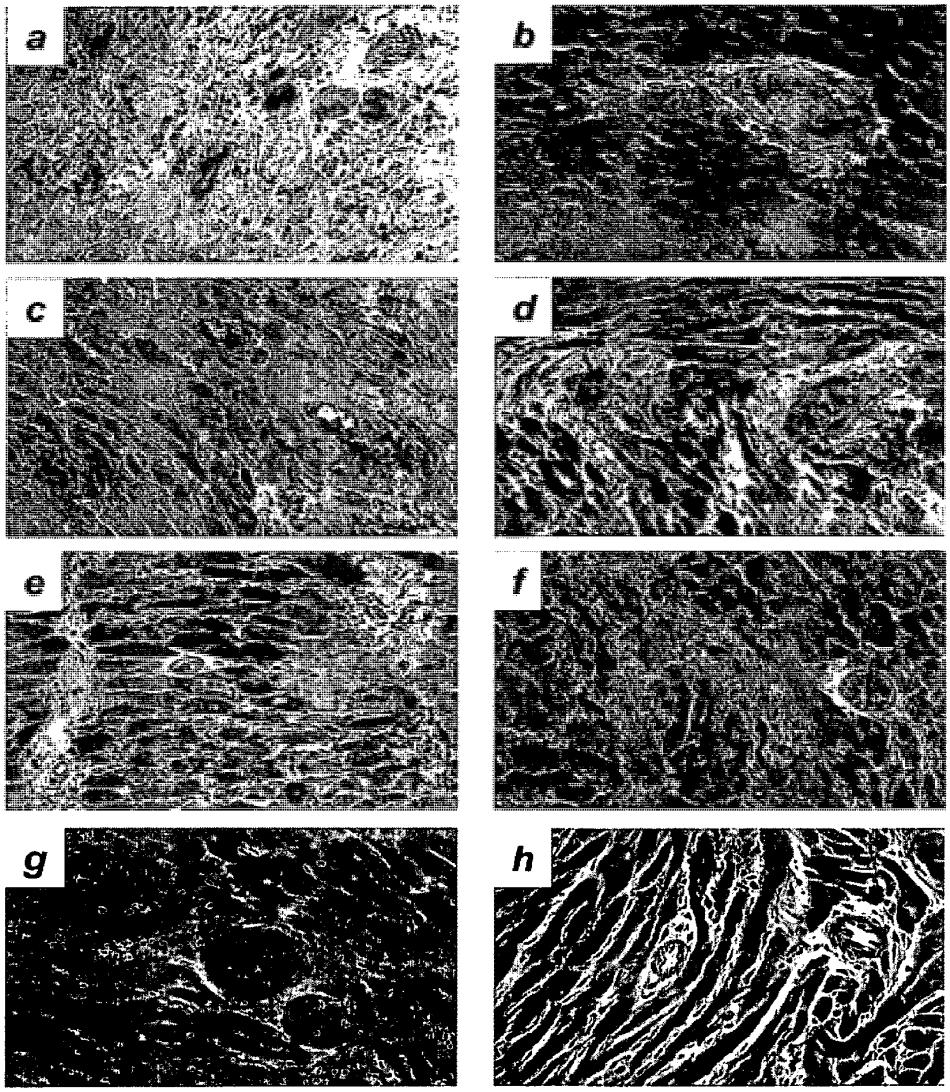


Figure 2B

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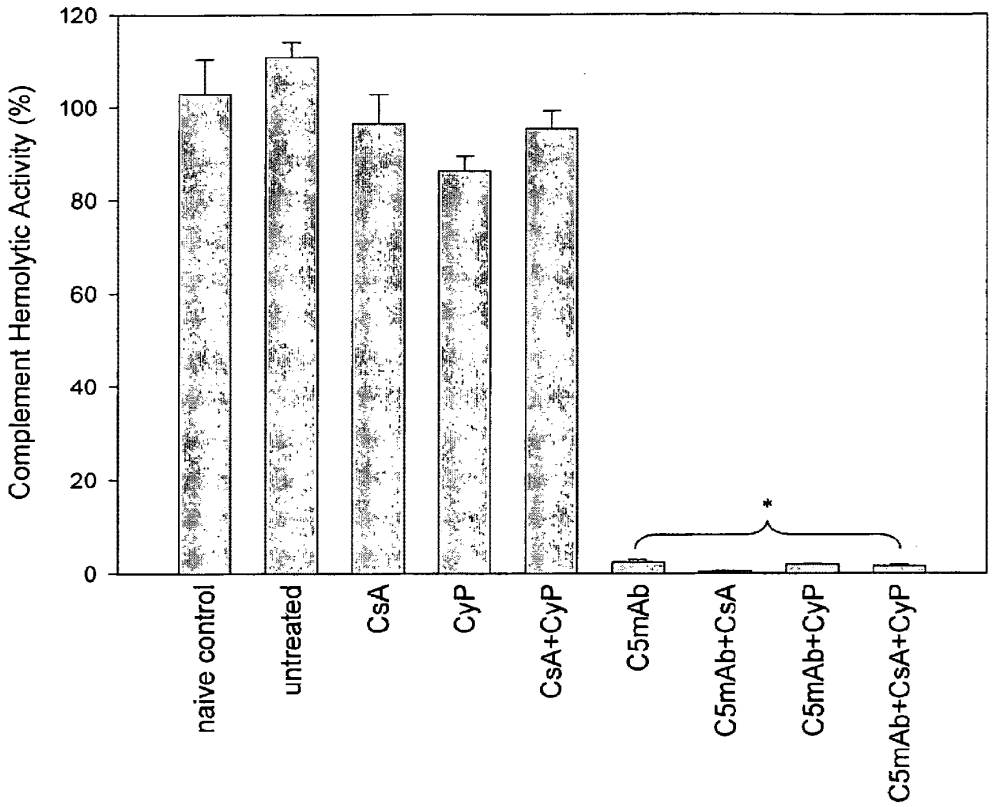


Figure 3

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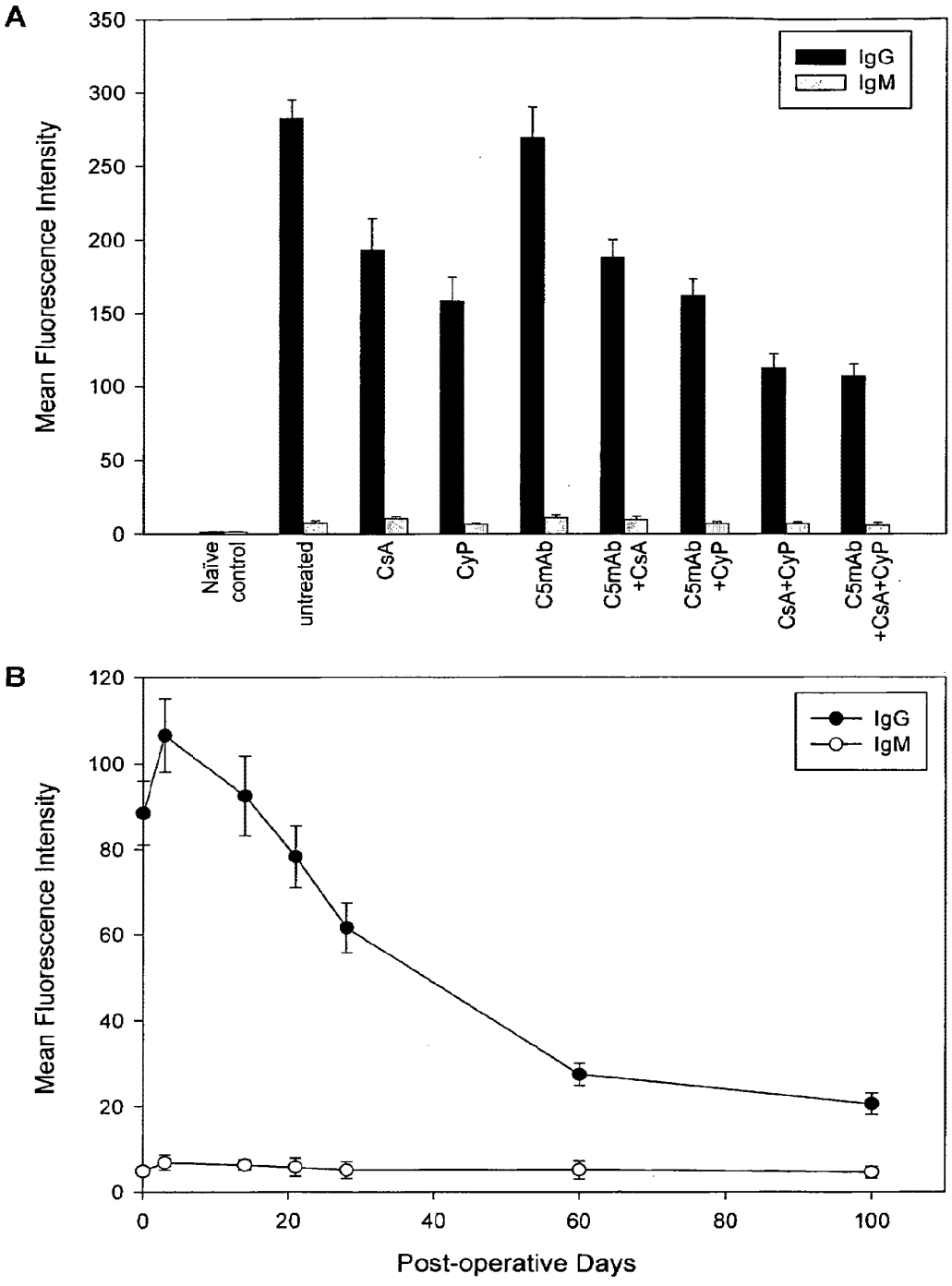
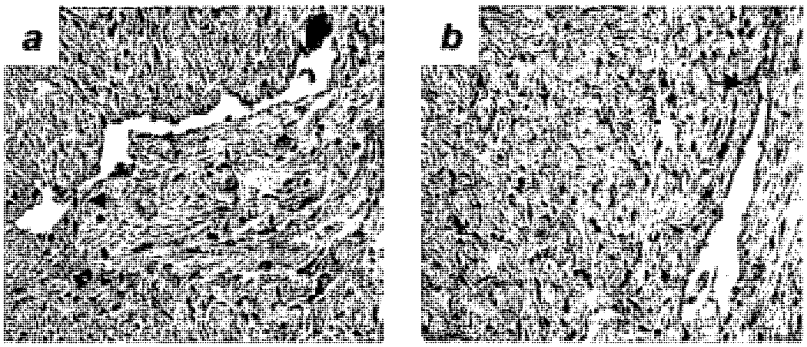


Figure 4A, B

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C



D

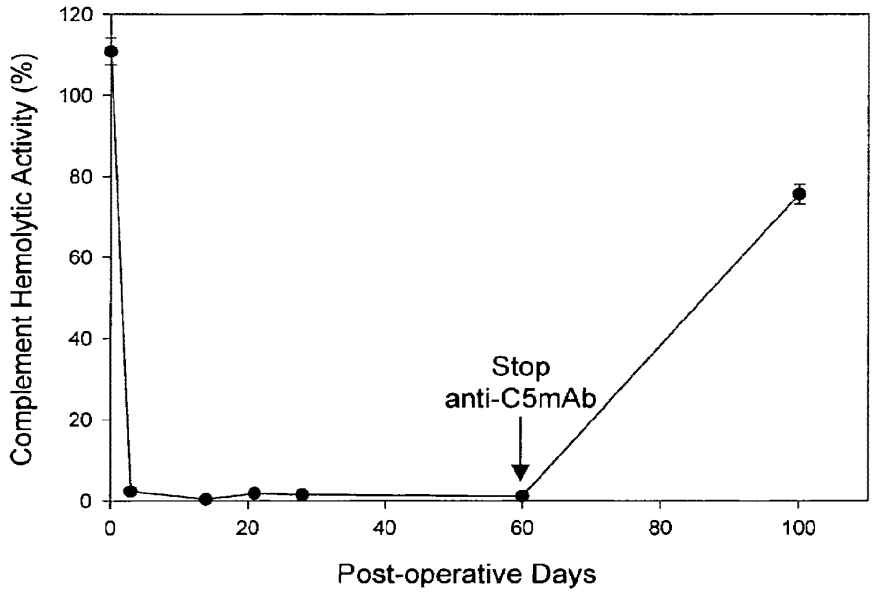


Figure 4C, D

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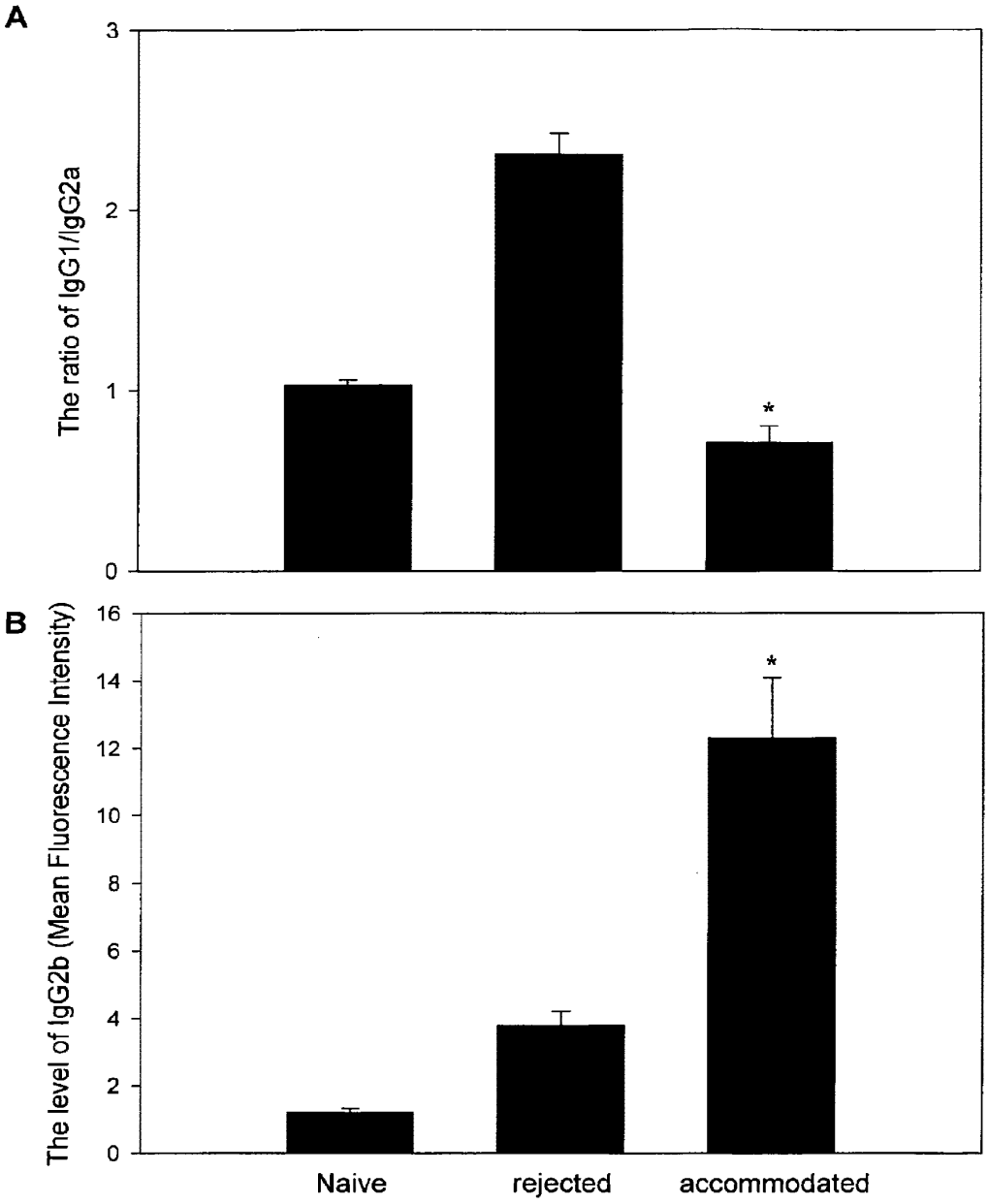


Figure 5A, B

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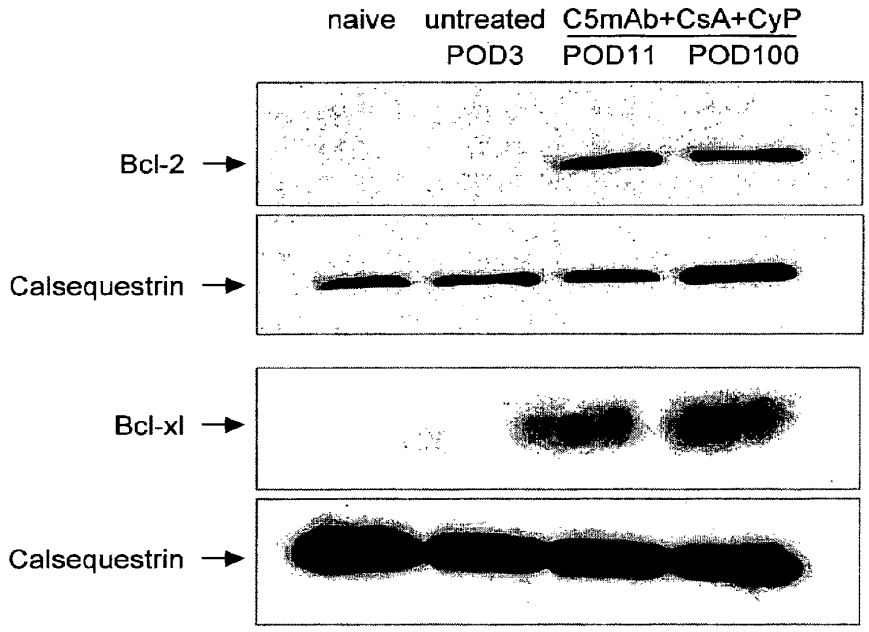


Figure 6

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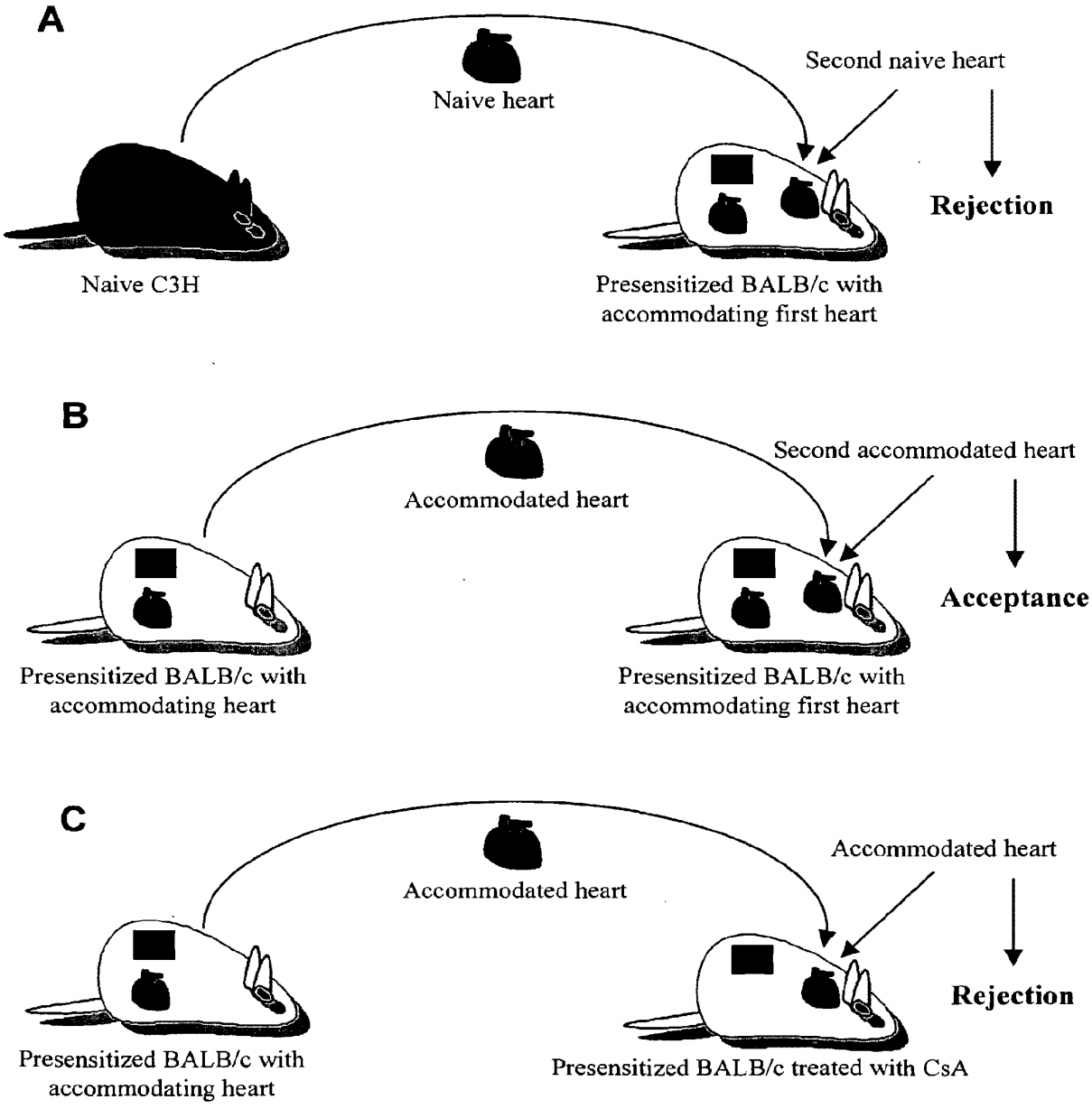
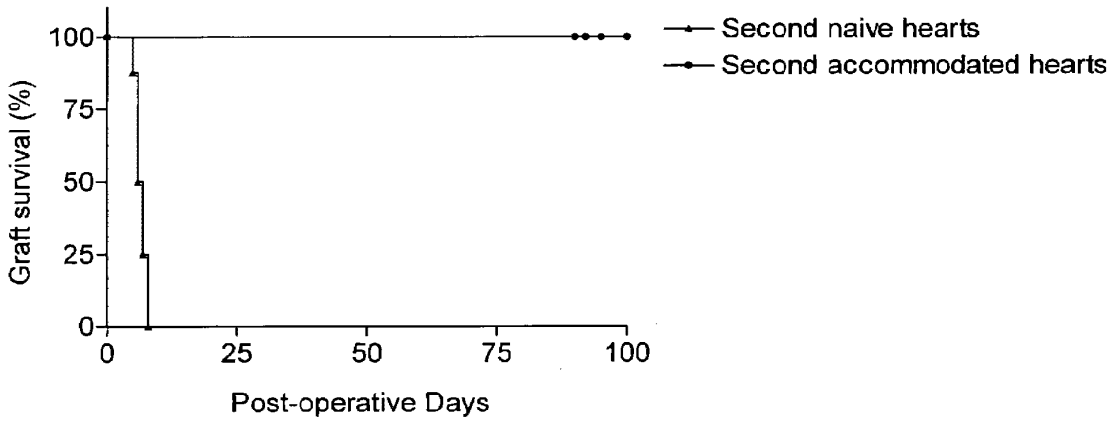


Figure 7

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A



B

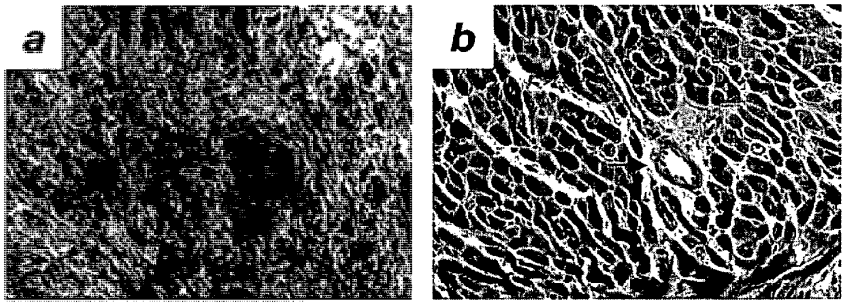


Figure 8

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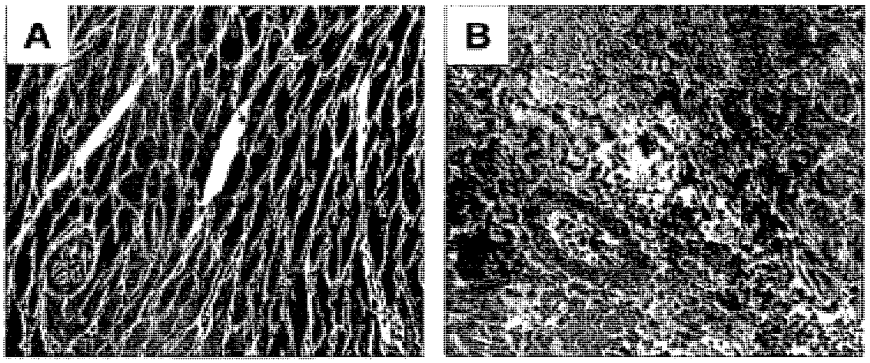


Figure 9

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(71) Applicant *(for all designated States except US)*: **XENCOR** [US/US]; 111 West Lemon Avenue, Monrovia, CA 91016 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LAZAR, Gregory, Alan** [US/US]; 268 W. Dryden Street, #114, Glendale, CA 91202 (US). **CHIRINO, Arthur, J.** [US/US]; 6816 Pueblo Vista, Camarillo, CA 91106 (US). **DANG, Wei** [CN/US]; 1111 Blanche Street, Apt. 312, Pasadena, CA 91106 (US). **DESJARLAIS, John, Rudolph** [US/US]; 2096 E. Crary Street, Pasadena, CA 91104 (US). **DOBERSTEIN, Stephen, Kohn** [US/US]; 700 S. Lake Avenue, #202, Pasadena, CA 91106 (US). **HAYES, Robert, J.** [GB/US]; 190 Arroyo Terrace, Pasadena, CA 91103 (US). **KARKI, Sher, Basudur** [NP/US]; 300 North El Molino Avenue, Pasadena, CA 91101 (US). **VAFA, Omid** [IR/US]; 724 Ocean View Avenue, Monrovia, CA 91016 (US).

(74) Agents: **SILVA, Robin, M.** et al.; DORSEY & WHITNEY LLP, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111 (US).

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

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(54) Title: OPTIMIZED FC VARIANTS AND METHODS FOR THEIR GENERATION

(57) Abstract: The present invention relates to optimized Fc variants, methods for their generation, and antibodies and Fc fusions comprising optimized Fc variants.

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OPTIMIZED Fc VARIANTS AND METHODS FOR THEIR GENERATION

[001] This application claims benefit under 35 U.S.C. §199(e) to USSNs 60/477,839 filed June 12, 2003; 60/467,606, filed May 2, 2003; 60/414,433 filed September 27, 2002; and 60/442,301 filed January 23, 2003, all of which are expressly incorporated by reference in their entirety.

FIELD OF THE INVENTION

[002] The present invention relates to novel optimized Fc variants, engineering methods for their generation, and their application, particularly for therapeutic purposes.

BACKGROUND OF THE INVENTION

[003] Antibodies are immunological proteins that bind a specific antigen. In most mammals, including humans and mice, antibodies are constructed from paired heavy and light polypeptide chains. Each chain is made up of individual immunoglobulin (Ig) domains, and thus the generic term immunoglobulin is used for such proteins. Each chain is made up of two distinct regions, referred to as the variable and constant regions. The light and heavy chain variable regions show significant sequence diversity between antibodies, and are responsible for binding the target antigen. The constant regions show less sequence diversity, and are responsible for binding a number of natural proteins to elicit important biochemical events. In humans there are five different classes of antibodies including IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The distinguishing features between these antibody classes are their constant regions, although subtler differences may exist in the V region. Figure 1 shows an IgG1 antibody, used here as an example to describe the general structural features of immunoglobulins. IgG antibodies are tetrameric proteins composed of two heavy chains and two light chains. The IgG heavy chain is composed of four immunoglobulin domains linked from N- to C-terminus in the order V_H-Cγ1-Cγ2-Cγ3, referring to the heavy chain variable domain, constant gamma 1 domain, constant gamma 2 domain, and constant gamma 3 domain respectively. The IgG light chain is composed of two immunoglobulin domains linked from N- to C-terminus in the order V_L-C_L, referring to the light chain variable domain and the light chain constant domain respectively.

[004] The variable region of an antibody contains the antigen binding determinants of the molecule, and thus determines the specificity of an antibody for its target antigen. The variable region is so named because it is the most distinct in sequence from other antibodies within the same class. The majority of sequence variability occurs in the complementarity determining regions (CDRs). There are 6 CDRs total, three each per heavy and light chain, designated V_H CDR1, V_H CDR2, V_H CDR3, V_L

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CDR1, V_L CDR2, and V_L CDR3. The variable region outside of the CDRs is referred to as the framework (FR) region. Although not as diverse as the CDRs, sequence variability does occur in the FR region between different antibodies. Overall, this characteristic architecture of antibodies provides a stable scaffold (the FR region) upon which substantial antigen binding diversity (the CDRs) can be explored by the immune system to obtain specificity for a broad array of antigens. A number of high-resolution structures are available for a variety of variable region fragments from different organisms, some unbound and some in complex with antigen. The sequence and structural features of antibody variable regions are well characterized (Morea *et al.*, 1997, *Biophys Chem* 68:9-16; Morea *et al.*, 2000, *Methods* 20:267-279), and the conserved features of antibodies have enabled the development of a wealth of antibody engineering techniques (Maynard *et al.*, 2000, *Annu Rev Biomed Eng* 2:339-376). For example, it is possible to graft the CDRs from one antibody, for example a murine antibody, onto the framework region of another antibody, for example a human antibody. This process, referred to in the art as "humanization", enables generation of less immunogenic antibody therapeutics from nonhuman antibodies. Fragments comprising the variable region can exist in the absence of other regions of the antibody, including for example the antigen binding fragment (Fab) comprising V_H-Cγ1 and V_H-C_L, the variable fragment (Fv) comprising V_H and V_L, the single chain variable fragment (scFv) comprising V_H and V_L linked together in the same chain, as well as a variety of other variable region fragments (Little *et al.*, 2000, *Immunol Today* 21:364-370).

[005] The Fc region of an antibody interacts with a number of Fc receptors and ligands, imparting an array of important functional capabilities referred to as effector functions. For IgG the Fc region, as shown in Figure 1, comprises Ig domains Cγ2 and Cγ3 and the N-terminal hinge leading into Cγ2. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system (Raghavan *et al.*, 1996, *Annu Rev Cell Dev Biol* 12:181-220; Ravetch *et al.*, 2001, *Annu Rev Immunol* 19:275-290). In humans this protein family includes FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2) (Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65). These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These receptors are expressed in a variety of immune cells including monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and $\gamma\gamma$ T cells. Formation of the Fc/FcγR complex recruits these effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack. The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell-mediated reaction wherein nonspecific cytotoxic

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cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Raghavan *et al.*, 1996, *Annu Rev Cell Dev Biol* 12:181-220; Ghetie *et al.*, 2000, *Annu Rev Immunol* 18:739-766; Ravetch *et al.*, 2001, *Annu Rev Immunol* 19:275-290). The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell is referred to as antibody dependent cell-mediated phagocytosis (ADCP). A number of structures have been solved of the extracellular domains of human FcγRs, including FcγRIIa (pdb accession code 1H9V)(Sondermann *et al.*, 2001, *J Mol Biol* 309:737-749) (pdb accession code 1FCG)(Maxwell *et al.*, 1999, *Nat Struct Biol* 6:437-442), FcγRIIb (pdb accession code 2FCB)(Sondermann *et al.*, 1999, *Embo J* 18:1095-1103); and FcγRIIIb (pdb accession code 1E4J)(Sondermann *et al.*, 2000, *Nature* 406:267-273.). All FcγRs bind the same region on Fc, at the N-terminal end of the Cγ2 domain and the preceding hinge, shown in Figure 2. This interaction is well characterized structurally (Sondermann *et al.*, 2001, *J Mol Biol* 309:737-749), and several structures of the human Fc bound to the extracellular domain of human FcγRIIIb have been solved (pdb accession code 1E4K)(Sondermann *et al.*, 2000, *Nature* 406:267-273.) (pdb accession codes 1IIS and 1IIX)(Radaev *et al.*, 2001, *J Biol Chem* 276:16469-16477), as well as has the structure of the human IgE Fc/Fc□RI□ complex (pdb accession code 1F6A)(Garman *et al.*, 2000, *Nature* 406:259-266).

[006] The different IgG subclasses have different affinities for the FcγRs, with IgG1 and IgG3 typically binding substantially better to the receptors than IgG2 and IgG4 (Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65). All FcγRs bind the same region on IgG Fc, yet with different affinities: the high affinity binder FcγRI has a Kd for IgG1 of 10^{-8} M^{-1}, whereas the low affinity receptors FcγRII and FcγRIII generally bind at 10^{-6} and 10^{-5} respectively. The extracellular domains of FcγRIIIa and FcγRIIIb are 96% identical, however FcγRIIIb does not have a intracellular signaling domain. Furthermore, whereas FcγRI, FcγRIIa/c, and FcγRIIIa are positive regulators of immune complex-triggered activation, characterized by having an intracellular domain that has an immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIb has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. Thus the former are referred to as activation receptors, and FcγRIIb is referred to as an inhibitory receptor. The receptors also differ in expression pattern and levels on different immune cells. Yet another level of complexity is the existence of a number of FcγR polymorphisms in the human proteome. A particularly relevant polymorphism with clinical significance is V158/F158 FcγRIIIa. Human IgG1 binds with greater affinity to the V158 allotype than to the F158 allotype. This difference in affinity, and presumably its effect on ADCC and/or ADCP, has been shown to be a significant determinant of the efficacy of the anti-CD20 antibody rituximab (Rituxan®, a registered trademark of IDEC Pharmaceuticals Corporation). Patients with the V158 allotype respond favorably to rituximab treatment; however, patients with the lower affinity F158 allotype respond poorly (Cartron *et al.*, 2002, *Blood* 99:754-758). Approximately 10-20% of humans are V158/V158

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homozygous, 45% are V158/F158 heterozygous, and 35-45% of humans are F158/F158 homozygous (Lehrnbecher *et al.*, 1999, *Blood* 94:4220-4232; Cartron *et al.*, 2002, *Blood* 99:754-758). Thus 80-90% of humans are poor responders, that is they have at least one allele of the F158 FcγRIIIa.

[007] An overlapping but separate site on Fc, shown in Figure 1, serves as the interface for the complement protein C1q. In the same way that Fc/FcγR binding mediates ADCC, Fc/C1q binding mediates complement dependent cytotoxicity (CDC). C1q forms a complex with the serine proteases C1r and C1s to form the C1 complex. C1q is capable of binding six antibodies, although binding to two IgGs is sufficient to activate the complement cascade. Similar to Fc interaction with FcγRs, different IgG subclasses have different affinity for C1q, with IgG1 and IgG3 typically binding substantially better to the FcγRs than IgG2 and IgG4 (Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65). There is currently no structure available for the Fc/C1q complex; however, mutagenesis studies have mapped the binding site on human IgG for C1q to a region involving residues D270, K322, K326, P329, and P331, and E333 (Idusogie *et al.*, 2000, *J Immunol* 164:4178-4184; Idusogie *et al.*, 2001, *J Immunol* 166:2571-2575).

[008] A site on Fc between the Cγ2 and Cγ3 domains, shown in Figure 1, mediates interaction with the neonatal receptor FcRn, the binding of which recycles endocytosed antibody from the endosome back to the bloodstream (Raghavan *et al.*, 1996, *Annu Rev Cell Dev Biol* 12:181-220; Ghetie *et al.*, 2000, *Annu Rev Immunol* 18:739-766). This process, coupled with preclusion of kidney filtration due to the large size of the full length molecule, results in favorable antibody serum half-lives ranging from one to three weeks. Binding of Fc to FcRn also plays a key role in antibody transport. The binding site for FcRn on Fc is also the site at which the bacterial proteins A and G bind. The tight binding by these proteins is typically exploited as a means to purify antibodies by employing protein A or protein G affinity chromatography during protein purification. Thus the fidelity of this region on Fc is important for both the clinical properties of antibodies and their purification. Available structures of the rat Fc/FcRn complex (Martin *et al.*, 2001, *Mol Cell* 7:867-877), and of the complexes of Fc with proteins A and G (Deisenhofer, 1981, *Biochemistry* 20:2361-2370; Sauer-Eriksson *et al.*, 1995, *Structure* 3:265-278; Tashiro *et al.*, 1995, *Curr Opin Struct Biol* 5:471-481) provide insight into the interaction of Fc with these proteins.

[009] A key feature of the Fc region is the conserved N-linked glycosylation that occurs at N297, shown in Figure 1. This carbohydrate, or oligosaccharide as it is sometimes referred, plays a critical structural and functional role for the antibody, and is one of the principle reasons that antibodies must be produced using mammalian expression systems. While not wanting to be limited to one theory, it is believed that the structural purpose of this carbohydrate may be to stabilize or solubilize Fc, determine a specific angle or level of flexibility between the Cγ3 and Cγ2 domains, keep the two Cγ2 domains from aggregating with one another across the central axis, or a combination of these.

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Efficient Fc binding to FcγR and C1q requires this modification, and alterations in the composition of the N297 carbohydrate or its elimination affect binding to these proteins (Umaña *et al.*, 1999, *Nat Biotechnol* 17:176-180; Davies *et al.*, 2001, *Biotechnol Bioeng* 74:288-294; Mimura *et al.*, 2001, *J Biol Chem* 276:45539-45547.; Radaev *et al.*, 2001, *J Biol Chem* 276:16478-16483; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740; Simmons *et al.*, 2002, *J Immunol Methods* 263:133-147). Yet the carbohydrate makes little if any specific contact with FcγRs (Radaev *et al.*, 2001, *J Biol Chem* 276:16469-16477), indicating that the functional role of the N297 carbohydrate in mediating Fc/FcγR binding may be via the structural role it plays in determining the Fc conformation. This is supported by a collection of crystal structures of four different Fc glycoforms, which show that the composition of the oligosaccharide impacts the conformation of Cγ2 and as a result the Fc/FcγR interface (Krapp *et al.*, 2003, *J Mol Biol* 325:979-989).

[010] The features of antibodies discussed above - specificity for target, ability to mediate immune effector mechanisms, and long half-life in serum - make antibodies powerful therapeutics. Monoclonal antibodies are used therapeutically for the treatment of a variety of conditions including cancer, inflammation, and cardiovascular disease. There are currently over ten antibody products on the market and hundreds in development. In addition to antibodies, an antibody-like protein that is finding an expanding role in research and therapy is the Fc fusion (Chamow *et al.*, 1996, *Trends Biotechnol* 14:52-60; Ashkenazi *et al.*, 1997, *Curr Opin Immunol* 9:195-200). An Fc fusion is a protein wherein one or more polypeptides is operably linked to Fc. An Fc fusion combines the Fc region of an antibody, and thus its favorable effector functions and pharmacokinetics, with the target-binding region of a receptor, ligand, or some other protein or protein domain. The role of the latter is to mediate target recognition, and thus it is functionally analogous to the antibody variable region. Because of the structural and functional overlap of Fc fusions with antibodies, the discussion on antibodies in the present invention extends directly to Fc fusions.

[011] Despite such widespread use, antibodies are not optimized for clinical use. Two significant deficiencies of antibodies are their suboptimal anticancer potency and their demanding production requirements. These deficiencies are addressed by the present invention

[012] There are a number of possible mechanisms by which antibodies destroy tumor cells, including anti-proliferation via blockage of needed growth pathways, intracellular signaling leading to apoptosis, enhanced down regulation and/or turnover of receptors, CDC, ADCC, ADCP, and promotion of an adaptive immune response (Cragg *et al.*, 1999, *Curr Opin Immunol* 11:541-547; Glennie *et al.*, 2000, *Immunol Today* 21:403-410). Anti-tumor efficacy may be due to a combination of these mechanisms, and their relative importance in clinical therapy appears to be cancer dependent. Despite this arsenal of anti-tumor weapons, the potency of antibodies as anti-cancer agents is unsatisfactory, particularly given their high cost. Patient tumor response data show that monoclonal

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antibodies provide only a small improvement in therapeutic success over normal single-agent cytotoxic chemotherapeutics. For example, just half of all relapsed low-grade non-Hodgkin's lymphoma patients respond to the anti-CD20 antibody rituximab (McLaughlin *et al.*, 1998, *J Clin Oncol* 16:2825-2833). Of 166 clinical patients, 6% showed a complete response and 42% showed a partial response, with median response duration of approximately 12 months. Trastuzumab (Herceptin®, a registered trademark of Genentech), an anti-HER2/neu antibody for treatment of metastatic breast cancer, has less efficacy. The overall response rate using trastuzumab for the 222 patients tested was only 15%, with 8 complete and 26 partial responses and a median response duration and survival of 9 to 13 months (Cobleigh *et al.*, 1999, *J Clin Oncol* 17:2639-2648). Currently for anticancer therapy, any small improvement in mortality rate defines success. Thus there is a significant need to enhance the capacity of antibodies to destroy targeted cancer cells.

[013] A promising means for enhancing the anti-tumor potency of antibodies is via enhancement of their ability to mediate cytotoxic effector functions such as ADCC, ADCP, and CDC. The importance of FcγR-mediated effector functions for the anti-cancer activity of antibodies has been demonstrated in mice (Clynes *et al.*, 1998, *Proc Natl Acad Sci U S A* 95:652-656; Clynes *et al.*, 2000, *Nat Med* 6:443-446), and the affinity of interaction between Fc and certain FcγRs correlates with targeted cytotoxicity in cell-based assays (Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Presta *et al.*, 2002, *Biochem Soc Trans* 30:487-490; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740). Additionally, a correlation has been observed between clinical efficacy in humans and their allotype of high (V158) or low (F158) affinity polymorphic forms of FcγRIIIa (Cartron *et al.*, 2002, *Blood* 99:754-758). Together these data suggest that an antibody with an Fc region optimized for binding to certain FcγRs may better mediate effector functions and thereby destroy cancer cells more effectively in patients. The balance between activating and inhibiting receptors is an important consideration, and optimal effector function may result from an Fc with enhanced affinity for activation receptors, for example FcγRI, FcγRIIa/c, and FcγRIIIa, yet reduced affinity for the inhibitory receptor FcγRIIb. Furthermore, because FcγRs can mediate antigen uptake and processing by antigen presenting cells, enhanced Fc/FcγR affinity may also improve the capacity of antibody therapeutics to elicit an adaptive immune response.

[014] Mutagenesis studies have been carried out on Fc towards various goals, with substitutions typically made to alanine (referred to as alanine scanning) or guided by sequence homology substitutions (Duncan *et al.*, 1988, *Nature* 332:563-564; Lund *et al.*, 1991, *J Immunol* 147:2657-2662; Lund *et al.*, 1992, *Mol Immunol* 29:53-59; Jefferis *et al.*, 1995, *Immunol Lett* 44:111-117; Lund *et al.*, 1995, *Faseb J* 9:115-119; Jefferis *et al.*, 1996, *Immunol Lett* 54:101-104; Lund *et al.*, 1996, *J Immunol* 157:4963-4969; Armour *et al.*, 1999, *Eur J Immunol* 29:2613-2624; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65) (US 5,624,821; US 5,885,573; PCT WO 00/42072; PCT WO 99/58572). The majority of substitutions reduce or ablate binding with FcγRs.

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However some success has been achieved at obtaining Fc variants with higher FcγR affinity. (See for example US 5,624,821, and PCT WO 00/42072). For example, Winter and colleagues substituted the human amino acid at position 235 of mouse IgG2b antibody (a glutamic acid to leucine mutation) that increased binding of the mouse antibody to human FcγRI by100-fold (Duncan *et al.*, 1988, *Nature* 332:563-564) (US 5,624,821). Shields *et al.* used alanine scanning mutagenesis to map Fc residues important to FcγR binding, followed by substitution of select residues with non-alanine mutations (Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Presta *et al.*, 2002, *Biochem Soc Trans* 30:487-490) (PCT WO 00/42072). Several mutations disclosed in this study, including S298A, E333A, and K334A, show enhanced binding to the activating receptor FcγRIIIa and reduced binding to the inhibitory receptor FcγRIIb. These mutations were combined to obtain double and triple mutation variants that show additive improvements in binding. The best variant disclosed in this study is a S298A/E333A/K334A triple mutant with approximately a 1.7-fold increase in binding to F158 FcγRIIIa, a 5-fold decrease in binding to FcγRIIb, and a 2.1-fold enhancement in ADCC.

[015] Enhanced affinity of Fc for FcγR has also been achieved using engineered glycoforms generated by expression of antibodies in engineered or variant cell lines (Umaña *et al.*, 1999, *Nat Biotechnol* 17:176-180; Davies *et al.*, 2001, *Biotechnol Bioeng* 74:288-294; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740; Shinkawa *et al.*, 2003, *J Biol Chem* 278:3466-3473). This approach has generated substantial enhancements of the capacity of antibodies to bind FcγRIIIa and to mediate ADCC. Although there are practical limitations such as the growth efficiency of the expression strains under large scale production conditions, this approach for enhancing Fc/FcγR affinity and effector function is promising. Indeed, coupling of these alternate glycoform technologies with the Fc variants of the present invention may provide additive or synergistic effects for optimal effector function.

[016] Although there is a need for greater effector function, for some antibody therapeutics reduced or eliminated effector function may be desired. This is often the case for therapeutic antibodies whose mechanism of action involves blocking or antagonism but not killing of the cells bearing target antigen. In these cases depletion of target cells is undesirable and can be considered a side effect. For example, the ability of anti-CD4 antibodies to block CD4 receptors on T cells makes them effective anti-inflammatories, yet their ability to recruit FcγR receptors also directs immune attack against the target cells, resulting in T cell depletion (Reddy *et al.*, 2000, *J Immunol* 164:1925-1933). Effector function can also be a problem for radiolabeled antibodies, referred to as radioconjugates, and antibodies conjugated to toxins, referred to as immunotoxins. These drugs can be used to destroy cancer cells, but the recruitment of immune cells via Fc interaction with FcγRs brings healthy immune cells in proximity to the deadly payload (radiation or toxin), resulting in depletion of normal lymphoid tissue along with targeted cancer cells (Hutchins *et al.*, 1995, *Proc Natl Acad Sci U S A* 92:11980-11984; White *et al.*, 2001, *Annu Rev Med* 52:125-145). This problem can potentially be circumvented by using IgG isotypes that poorly recruit complement or effector cells, for example IgG2

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and IgG4. An alternate solution is to develop Fc variants that reduce or ablate binding (Alegre *et al.*, 1994, *Transplantation* 57:1537-1543; Hutchins *et al.*, 1995, *Proc Natl Acad Sci U S A* 92:11980-11984; Armour *et al.*, 1999, *Eur J Immunol* 29:2613-2624; Reddy *et al.*, 2000, *J Immunol* 164:1925-1933; Xu *et al.*, 2000, *Cell Immunol* 200:16-26; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604) (US 6,194,551; US 5,885,573; PCT WO 99/58572). A critical consideration for the reduction or elimination of effector function is that other important antibody properties not be perturbed. Fc variants should be engineered that not only ablate binding to FcγRs and/or C1q, but also maintain antibody stability, solubility, and structural integrity, as well as ability to interact with other important Fc ligands such as FcRn and proteins A and G.

[017] The present invention addresses another major shortcoming of antibodies, namely their demanding production requirements (Garber, 2001, *Nat Biotechnol* 19:184-185; Dove, 2002, *Nat Biotechnol* 20:777-779). Antibodies must be expressed in mammalian cells, and the currently marketed antibodies together with other high-demand biotherapeutics consume essentially all of the available manufacturing capacity. With hundreds of biologics in development, the majority of which are antibodies, there is an urgent need for more efficient and cheaper methods of production. The downstream effects of insufficient antibody manufacturing capacity are three-fold. First, it dramatically raises the cost of goods to the producer, a cost that is passed on to the patient. Second, it hinders industrial production of approved antibody products, limiting availability of high demand therapeutics to patients. Finally, because clinical trials require large amounts of a protein that is not yet profitable, the insufficient supply impedes progress of the growing antibody pipeline to market.

[018] Alternative production methods have been explored in attempts at alleviating this problem. Transgenic plants and animals are being pursued as potentially cheaper and higher capacity production systems (Chadd *et al.*, 2001, *Curr Opin Biotechnol* 12:188-194). Such expression systems, however, can generate glycosylation patterns significantly different from human glycoproteins. This may result in reduced or even lack of effector function because, as discussed above, the carbohydrate structure can significantly impact FcγR and complement binding. A potentially greater problem with nonhuman glycoforms may be immunogenicity; carbohydrates are a key source of antigenicity for the immune system, and the presence of nonhuman glycoforms has a significant chance of eliciting antibodies that neutralize the therapeutic, or worse cause adverse immune reactions. Thus the efficacy and safety of antibodies produced by transgenic plants and animals remains uncertain. Bacterial expression is another attractive solution to the antibody production problem. Expression in bacteria, for example *E. coli*, provides a cost-effective and high capacity method for producing proteins. For complex proteins such as antibodies there are a number of obstacles to bacterial expression, including folding and assembly of these complex molecules, proper disulfide formation, and solubility, stability, and functionality in the absence of glycosylation because proteins expressed in bacteria are not glycosylated. Full length unglycosylated antibodies that bind antigen have been successfully expressed in *E. coli* (Simmons *et al.*, 2002, *J Immunol*

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Methods 263:133-147), and thus, folding, assembly, and proper disulfide formation of bacterially expressed antibodies are possible in the absence of the eukaryotic chaperone machinery. However the ultimate utility of bacterially expressed antibodies as therapeutics remains hindered by the lack of glycosylation, which results in lack effector function and may result in poor stability and solubility. This will likely be more problematic for formulation at the high concentrations for the prolonged periods demanded by clinical use.

[019] An aglycosylated Fc with favorable solution properties and the capacity to mediate effector functions would be significantly enabling for the alternate production methods described above. By overcoming the structural and functional shortcomings of aglycosylated Fc, antibodies can be produced in bacteria and transgenic plants and animals with reduced risk of immunogenicity, and with effector function for clinical applications in which cytotoxicity is desired such as cancer. The present invention describes the utilization of protein engineering methods to develop stable, soluble Fc variants with effector function. Currently, such Fc variants do not exist in the art.

[020] In summary, there is a need for antibodies with enhanced therapeutic properties. Engineering of optimized or enhanced Fc variants is a promising approach to meeting this need. Yet a substantial obstacle to engineering Fc variants with the desired properties is the difficulty in predicting what amino acid modifications, out of the enormous number of possibilities, will achieve the desired goals, coupled with the inefficient production and screening methods for antibodies. Indeed one of the principle reasons for the incomplete success of the prior art is that approaches to Fc engineering have thus far involved hit-or-miss methods such as alanine scans or production of glycoforms using different expression strains. In these studies, the Fc modifications that were made were fully or partly random in hopes of obtaining variants with favorable properties. The present invention provides a variety of engineering methods, many of which are based on more sophisticated and efficient techniques, which may be used to overcome these obstacles in order to develop Fc variants that are optimized for the desired properties. The described engineering methods provide design strategies to guide Fc modification, computational screening methods to design favorable Fc variants, library generation approaches for determining promising variants for experimental investigation, and an array of experimental production and screening methods for determining the Fc variants with favorable properties.

SUMMARY OF THE INVENTION

[021] The present invention provides Fc variants that are optimized for a number of therapeutically relevant properties.

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[022] It is an object of the present invention to provide novel Fc positions at which amino acid modifications may be made to generate optimized Fc variants. Said Fc positions include 240, 244, 245, 247, 262, 263, 266, 299, 313, 325, 328, and 332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. The present invention describes any amino acid modification at any of said novel Fc positions in order to generate an optimized Fc variant.

[023] It is a further object of the present invention to provide Fc variants that have been screened computationally. A computationally screened Fc variant is one that is predicted by the computational screening calculations described herein as having a significantly greater potential than random for being optimized for a desired property. In this way, computational screening serves as a prelude to or surrogate for experimental screening, and thus said computationally screened Fc variants are considered novel.

[024] It is a further object of the present invention to provide Fc variants that have been characterized using one or more of the experimental methods described herein. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 327, 328, 329, 330, and 332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise at least one substitution selected from the group consisting of L234D, L234E, L234N, L234Q, L234T, L234H, L234Y, L234I, L234V, L234F, L235D, L235S, L235N, L235Q, L235T, L235H, L235Y, L235I, L235V, L235F, S239D, S239E, S239N, S239Q, S239F, S239T, S239H, S239Y, V240I, V240A, V240T, V240M, F241W, F241L, F241Y, F241E, F241R, F243W, F243L F243Y, F243R, F243Q, P244H, P245A, P247V, P247G, V262I, V262A, V262T, V262E, V263I, V263A, V263T, V263M, V264L, V264I, V264W, V264T, V264R, V264F, V264M, V264Y, V264E, D265G, D265N, D265Q, D265Y, D265F, D265V, D265I, D265L, D265H, D265T, V266I, V266A, V266T, V266M, S267Q, S267L, E269H, E269Y, E269F, E269R, Y296E, Y296Q, Y296D, Y296N, Y296S, Y296T, Y296L, Y296I, Y296H, N297S, N297D, N297E, A298H, T299I, T299L, T299A, T299S, T299V, T299H, T299F, T299E, W313F, N325Q, N325L, N325I, N325D, N325E, N325A, N325T, N325V, N325H, A327N, A327L, L328M, L328D, L328E, L328N, L328Q, L328F, L328I, L328V, L328T, L328H, L328A, P329F, A330L, A330Y, A330V, A330I, A330F, A330R, A330H, I332D, I332E, I332N, I332Q, I332T, I332H, I332Y, and I332A, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of V264L, V264I, F241W, F241L, F243W, F243L, F241L/F243L/V262I/V264I, F241W/F243W, F241W/F243W/V262A/V264A, F241L/V262I, F243L/V264I, F243L/V262I/V264W, F241Y/F243Y/V262T/V264T, F241E/F243R/V262E/V264R, F241E/F243Q/V262T/V264E, F241R/F243Q/V262T/V264R, F241E/F243Y/V262T/V264R, L328M, L328E, L328F, I332E, L328M/I332E, P244H, P245A, P247V, W313F, P244H/P245A/P247V, P247G, V264I/I332E, F241E/F243R/V262E/V264R/I332E, F241E/F243Q/V262T/V264E/I332E,

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F241R/F243Q/V262T/V264R/I332E, F241E/F243Y/V262T/V264R/I332E, S298A/I332E, S239E/I332E, S239Q/I332E, S239E, D265G, D265N, S239E/D265G, S239E/D265N, S239E/D265Q, Y296E, Y296Q, T299I, A327N, S267Q/A327S, S267L/A327S , A327L, P329F, A330L, A330Y, I332D, N297S, N297D, N297S/I332E, N297D/I332E, N297E/I332E, D265Y/N297D/I332E, D265Y/N297D/T299L/I332E, D265F/N297E/I332E, L328I/I332E, L328Q/I332E, I332N, I332Q, V264T, V264F, V240I, V263I, V266I, T299A, T299S, T299V, N325Q, N325L, N325I, S239D, S239N, S239F, S239D/I332D, S239D/I332E, S239D/I332N, S239D/I332Q, S239E/I332D, S239E/I332N, S239E/I332Q, S239N/I332D, S239N/I332E, S239N/I332N, S239N/I332Q, S239Q/I332D, S239Q/I332N, S239Q/I332Q, Y296D, Y296N, F241Y/F243Y/V262T/V264T/N297D/I332E, A330Y/I332E, V264I/A330Y/I332E, A330L/I332E, V264I/A330L/I332E, L234D, L234E, L234N, L234Q, L234T, L234H, L234Y, L234I, L234V, L234F, L235D, L235S, L235N, L235Q, L235T, L235H, L235Y, L235I, L235V, L235F, S239T, S239H, S239Y, V240A, V240T, V240M, V263A, V263T, V263M, V264M, V264Y, V266A, V266T, V266M, E269H, E269Y, E269F, E269R, Y296S, Y296T, Y296L, Y296I, A298H, T299H, A330V, A330I, A330F, A330R, A330H, N325D, N325E, N325A, N325T, N325V, N325H, L328D/I332E, L328E/I332E, L328N/I332E, L328Q/I332E, L328V/I332E, L328T/I332E, L328H/I332E, L328I/I332E, L328A, I332T, I332H, I332Y, I332A, S239E/V264I/I332E, S239Q/V264I/I332E, S239E/V264I/A330Y/I332E, S239E/V264I/S298A/A330Y/I332E, S239D/N297D/I332E, S239E/N297D/I332E, S239D/D265V/N297D/I332E, S239D/D265I/N297D/I332E, S239D/D265L/N297D/I332E, S239D/D265F/N297D/I332E, S239D/D265Y/N297D/I332E, S239D/D265H/N297D/I332E, S239D/D265T/N297D/I332E, V264E/N297D/I332E, Y296D/N297D/I332E, Y296E/N297D/I332E, Y296N/N297D/I332E, Y296Q/N297D/I332E, Y296H/N297D/I332E, Y296T/N297D/I332E, N297D/T299V/I332E, N297D/T299I/I332E, N297D/T299L/I332E, N297D/T299F/I332E, N297D/T299H/I332E, N297D/T299E/I332E, N297D/A330Y/I332E, N297D/S298A/A330Y/I332E, S239D/A330Y/I332E, S239N/A330Y/I332E, S239D/A330L/I332E, S239N/A330L/I332E, V264I/S298A/I332E, S239D/S298A/I332E, S239N/S298A/I332E, S239D/V264I/I332E, S239D/V264I/S298A/I332E, and S239D/V264I/A330L/I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[025] It is a further object of the present invention to provide an Fc variant that binds with greater affinity to one or more FcγRs. In one embodiment, said Fc variants have affinity for an FcγR that is more than 1-fold greater than that of the parent Fc polypeptide. In an alternate embodiment, said Fc variants have affinity for an FcγR that is more than 5-fold greater than that of the parent Fc polypeptide. In a preferred embodiment, said Fc variants have affinity for an FcγR that is between 5-fold and 300-fold greater than that of the parent Fc polypeptide. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 243, 264, 266, 328, 330, 332, and 325, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise at least one amino acid substitution selected from the group consisting of: L234E, L234Y, L234I, L235D,

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L235S, L235Y, L235I, S239D, S239E, S239N, S239Q, S239T, V240I, V240M, F243L, V264I, V264T, V264Y, V266I, L328M, L328I, L328Q, L328D, L328V, L328T, A330Y, A330L, A330I, I332D, I332E, I332N, I332Q, and N325T, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of V264I, F243L/V264I, L328M, I332E, L328M/I332E, V264I/I332E, S298A/I332E, S239E/I332E, S239Q/I332E, S239E, A330Y, I332D, L328I/I332E, L328Q/I332E, V264T, V240I, V266I, S239D, S239D/I332D, S239D/I332E, S239D/I332N, S239D/I332Q, S239E/I332D, S239E/I332N, S239E/I332Q, S239N/I332D, S239N/I332E, S239Q/I332D, A330Y/I332E, V264I/A330Y/I332E, A330L/I332E, V264I/A330L/I332E, L234E, L234Y, L234I, L235D, L235S, L235Y, L235I, S239T, V240M, V264Y, A330I, N325T, L328D/I332E, L328V/I332E, L328T/I332E, L328I/I332E, S239E/V264I/I332E, S239Q/V264I/I332E, S239E/V264I/A330Y/I332E, S239D/A330Y/I332E, S239N/A330Y/I332E, S239D/A330L/I332E, S239N/A330L/I332E, V264I/S298A/I332E, S239D/S298A/I332E, S239N/S298A/I332E, S239D/V264I/I332E, S239D/V264I/S298A/I332E, and S239D/V264I/A330L/I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[026] It is a further object of the present invention to provide Fc variantd that have a FcγRIIIa-fold:FcγRIIb-fold ratio greater than 1:1. In one embodiment, said Fc variants have a FcγRIIIa-fold:FcγRIIb-fold ratio greater than 11:1. In a preferred embodiment, said Fc variants have a FcγRIIIa-fold:FcγRIIb-fold ratio between 11:1 and 86:1. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 264, 296, 330, and I332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise at least one amino acid substitution selected from the group consisting of: L234Y, L234I, L235I, S239D, S239E, S239N, S239Q, V240A, V240M, V264I, V264Y, Y296Q, A330L, A330Y, A330I, I332D, and I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of: I332E, V264I/I332E, S239E/I332E, S239Q/I332E, Y296Q, A330L, A330Y, I332D, S239D, S239D/I332E, A330Y/I332E, V264I/A330Y/I332E, A330L/I332E, V264I/A330L/I332E, L234Y, L234I, L235I, V240A, V240M, V264Y, A330I, S239D/A330L/I332E, S239D/S298A/I332E, S239N/S298A/I332E, S239D/V264I/I332E, S239D/V264I/S298A/I332E, and S239D/V264I/A330L/I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[027] It is a further object of the present invention to provide Fc variants that mediate effector function more effectively in the presence of effector cells. In one embodiment, said Fc variants mediate ADCC that is greater than that mediated by the parent Fc polypeptide. In a preferred embodiment, said Fc variants mediate ADCC that is more than 5-fold greater than that mediated by the parent Fc polypeptide. In a mostly preferred embodiment, said Fc variants mediate ADCC that is between 5-fold and 50-fold greater than that mediated by the parent Fc polypeptide. In one

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embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 243, 264, 266, 328, 330, 332, and 325, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise at least one amino acid substitutions selected from the group consisting of: L234E, L234Y, L234I, L235D, L235S, L235Y, L235I, S239D, S239E, S239N, S239Q, S239T, V240I, V240M, F243L, V264I, V264T, V264Y, V266I, L328M, L328I, L328Q, L328D, L328V, L328T, A330Y, A330L, A330I, I332D, I332E, I332N, I332Q, and N325T, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of: V264I, F243L/V264I, L328M, I332E, L328M/I332E, V264I/I332E, S298A/I332E, S239E/I332E, S239Q/I332E, S239E, A330Y, I332D, L328I/I332E, L328Q/I332E, V264T, V240I, V266I, S239D, S239D/I332D, S239D/I332E, S239D/I332N, S239D/I332Q, S239E/I332D, S239E/I332N, S239E/I332Q, S239N/I332D, S239N/I332E, S239Q/I332D, A330Y/I332E, V264I/A330Y/I332E, A330L/I332E, V264I/A330L/I332E, L234E, L234Y, L234I, L235D, L235S, L235Y, L235I, S239T, V240M, V264Y, A330I, N325T, L328D/I332E, L328V/I332E, L328T/I332E, L328I/I332E, S239E/V264I/I332E, S239Q/V264I/I332E, S239E/V264I/A330Y/I332E, S239D/A330Y/I332E, S239N/A330Y/I332E, S239D/A330L/I332E, S239N/A330L/I332E, V264I/S298A/I332E, S239D/S298A/I332E, S239N/S298A/I332E, S239D/V264I/I332E, S239D/V264I/S298A/I332E, and S239D/V264I/A330L/I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[028] It is a further object of the present invention to provide Fc variants that bind with weaker affinity to one or more FcγRs. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 327, 328, 329, 330, and 332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise an amino acid substitution at a position selected from the group consisting of: L234D, L234N, L234Q, L234T, L234H, L234V, L234F, L235N, L235Q, L235T, L235H, L235V, L235F, S239E, S239N, S239Q, S239F, S239H, S239Y, V240A, V240T, F241W, F241L, F241Y, F241E, F241R, F243W, F243L F243Y, F243R, F243Q, P244H, P245A, P247V, P247G, V262I, V262A, V262T, V262E, V263I, V263A, V263T, V263M, V264L, V264I, V264W, V264T, V264R, V264F, V264M, V264E, D265G, D265N, D265Q, D265Y, D265F, D265V, D265I, D265L, D265H, D265T, V266A, V266T, V266M, S267Q, S267L, E269H, E269Y, E269F, E269R, Y296E, Y296Q, Y296D, Y296N, Y296S, Y296T, Y296L, Y296I, Y296H, N297S, N297D, N297E, A298H, T299I, T299L, T299A, T299S, T299V, T299H, T299F, T299E, W313F, N325Q, N325L, N325I, N325D, N325E, N325A, N325V, N325H, A327N, A327L, L328M, 328E, L328N, L328Q, L328F, L328H, L328A, P329F, A330L, A330V, A330F, A330R, A330H, I332N, I332Q, I332T, I332H, I332Y, and I332A, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of: V264L, F241W, F241L, F243W, F243L, F241L/F243L/V262I/V264I, F241W/F243W, F241W/F243W/V262A/V264A, F241L/V262I, F243L/V262I/V264W, F241Y/F243Y/V262T/V264T,

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F241E/F243R/V262E/V264R, F241E/F243Q/V262T/V264E, F241R/F243Q/V262T/V264R, F241E/F243Y/V262T/V264R, L328M, L328E, L328F, P244H, P245A, P247V, W313F, P244H/P245A/P247V, P247G, F241E/F243R/V262E/V264R/I332E, F241E/F243Y/V262T/V264R/I332E, D265G, D265N, S239E/D265G, S239E/D265N, S239E/D265Q, Y296E, Y296Q, T299I, A327N, S267Q/A327S, S267L/A327S , A327L, P329F, A330L, N297S, N297D, N297S/I332E, I332N, I332Q, V264F, V263I, T299A, T299S, T299V, N325Q, N325L, N325I, S239N, S239F, S239N/I332N, S239N/I332Q, S239Q/I332N, S239Q/I332Q, Y296D, Y296N, L234D, L234N, L234Q, L234T, L234H, L234V, L234F, L235N, L235Q, L235T, L235H, L235V, L235F, S239H, S239Y, V240A, V263T, V263M, V264M, V266A, V266T, V266M, E269H, E269Y, E269F, E269R, Y296S, Y296T, Y296L, Y296I, A298H, T299H, A330V, A330F, A330R, A330H, N325D, N325E, N325A, N325V, N325H, L328E/I332E, L328N/I332E, L328Q/I332E, L328H/I332E, L328A, I332T, I332H, I332Y, and I332A, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[029] It is a further object of the present invention to provide Fc variants that mediate ADCC in the presence of effector cells less effectively. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 327, 328, 329, 330, and 332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: L234D, L234N, L234Q, L234T, L234H, L234V, L234F, L235N, L235Q, L235T, L235H, L235V, L235F, S239E, S239N, S239Q, S239F, S239H, S239Y, V240A, V240T, F241W, F241L, F241Y, F241E, F241R, F243W, F243L F243Y, F243R, F243Q, P244H, P245A, P247V, P247G, V262I, V262A, V262T, V262E, V263I, V263A, V263T, V263M, V264L, V264I, V264W, V264T, V264R, V264F, V264M, V264E, D265G, D265N, D265Q, D265Y, D265F, D265V, D265I, D265L, D265H, D265T, V266A, V266T, V266M, S267Q, S267L, E269H, E269Y, E269F, E269R, Y296E, Y296Q, Y296D, Y296N, Y296S, Y296T, Y296L, Y296I, Y296H, N297S, N297D, N297E, A298H, T299I, T299L, T299A, T299S, T299V, T299H, T299F, T299E, W313F, N325Q, N325L, N325I, N325D, N325E, N325A, N325V, N325H, A327N, A327L, L328M, 328E, L328N, L328Q, L328F, L328H, L328A, P329F, A330L, A330V, A330F, A330R, A330H, I332N, I332Q, I332T, I332H, I332Y, and I332A, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of: V264L, F241W, F241L, F243W, F243L, F241L/F243L/V262I/V264I, F241W/F243W, F241W/F243W/V262A/V264A, F241L/V262I, F243L/V262I/V264W, F241Y/F243Y/V262T/V264T, F241E/F243R/V262E/V264R, F241E/F243Q/V262T/V264E, F241R/F243Q/V262T/V264R, F241E/F243Y/V262T/V264R, L328M, L328E, L328F, P244H, P245A, P247V, W313F, P244H/P245A/P247V, P247G, F241E/F243R/V262E/V264R/I332E, F241E/F243Y/V262T/V264R/I332E, D265G, D265N, S239E/D265G, S239E/D265N, S239E/D265Q, Y296E, Y296Q, T299I, A327N, S267Q/A327S, S267L/A327S , A327L, P329F, A330L, N297S, N297D, N297S/I332E, I332N, I332Q, V264F, V263I, T299A, T299S, T299V, N325Q, N325L, N325I,

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S239N, S239F, S239N/I332N, S239N/I332Q, S239Q/I332N, S239Q/I332Q, Y296D, Y296N, L234D, L234N, L234Q, L234T, L234H, L234V, L234F, L235N, L235Q, L235T, L235H, L235V, L235F, S239H, S239Y, V240A, V263T, V263M, V264M, V266A, V266T, V266M, E269H, E269Y, E269F, E269R, Y296S, Y296T, Y296L, Y296I, A298H, T299H, A330V, A330F, A330R, A330H, N325D, N325E, N325A, N325V, N325H, L328E/I332E, L328N/I332E, L328Q/I332E, L328H/I332E, L328A, I332T, I332H, I332Y, and I332A, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[030] It is a further object of the present invention to provide Fc variants that have improved function and/or solution properties as compared to the aglycosylated form of the parent Fc polypeptide. Improved functionality herein includes but is not limited to binding affinity to an Fc ligand. Improved solution properties herein includes but is not limited to stability and solubility. In one embodiment, said aglycosylated Fc variants bind to an FcγR with an affinity that is comparable to or better than the glycosylated parent Fc polypeptide. In an alternate embodiment, said Fc variants bind to an FcγR with an affinity that is within 0.4-fold of the glycosylated form of the parent Fc polypeptide. In one embodiment, said Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 239, 241, 243, 262, 264, 265, 296, 297, 330, and 332, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a preferred embodiment, said Fc variants comprise an amino acid substitution selected from the group consisting of: S239D, S239E, F241Y, F243Y, V262T, V264T, V264E, D265Y, D265H, Y296N, N297D, A330Y, and I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. In a mostly preferred embodiment, said Fc variants are selected from the group consisting of: N297D/I332E, F241Y/F243Y/V262T/V264T/N297D/I332E, S239D/N297D/I332E, S239E/N297D/I332E, S239D/D265Y/N297D/I332E, S239D/D265H/N297D/I332E, V264E/N297D/I332E, Y296N/N297D/I332E, and N297D/A330Y/I332E, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

[031] The present invention also provides methods for engineering optimized Fc variants. It is an object of the present invention to provide design strategies that may be used to guide Fc optimization. It is a further object of the present invention to provide computational screening methods that may be used to design Fc variants. It is a further object of the present invention to provide methods for generating libraries for experimental testing. It is a further object of the present invention to provide experimental production and screening methods for obtaining optimized Fc variants.

[032] The present invention provides isolated nucleic acids encoding the Fc variants described herein. The present invention provides vectors comprising said nucleic acids, optionally, operably linked to control sequences. The present invention provides host cells containing the vectors, and methods for producing and optionally recovering the Fc variants.

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[033] The present invention provides novel antibodies and Fc fusions that comprise the Fc variants disclosed herein. Said novel antibodies and Fc fusions may find use in a therapeutic product.

[034] The present invention provides compositions comprising antibodies and Fc fusions that comprise the Fc variants described herein, and a physiologically or pharmaceutically acceptable carrier or diluent.

[035] The present invention contemplates therapeutic and diagnostic uses for antibodies and Fc fusions that comprise the Fc variants disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[036] Figure 1. Antibody structure and function. Shown is a model of a full length human IgG1 antibody, modeled using a humanized Fab structure from pdb accession code 1CE1 (James *et al.*, 1999, *J Mol Biol* 289:293-301) and a human IgG1 Fc structure from pdb accession code 1DN2 (DeLano *et al.*, 2000, *Science* 287:1279-1283). The flexible hinge that links the Fab and Fc regions is not shown. IgG1 is a homodimer of heterodimers, made up of two light chains and two heavy chains. The Ig domains that comprise the antibody are labeled, and include V_L and C_L for the light chain, and V_H, Cgamma1 (Cγ1), Cgamma2 (Cγ2), and Cgamma3 (Cγ3) for the heavy chain. The Fc region is labeled. Binding sites for relevant proteins are labeled, including the antigen binding site in the variable region, and the binding sites for FcγRs, FcRn, C1q, and proteins A and G in the Fc region.

[037] Figure 2. The Fc/FcγRIIIb complex structure 1IIS. Fc is shown as a gray ribbon diagram, and FcγRIIIb is shown as a black ribbon. The N297 carbohydrate is shown as black sticks.

[038] Figure 3. The amino acid sequence of the heavy chain of the antibody alemtuzumab (Campath®, a registered trademark of Ilex Pharmaceuticals LP), illustrating positions numbered sequentially (2 lines above the amino acid sequence) and positions numbered according to the EU index as in Kabat (2 lines below the amino acid sequence. The approximate beginnings of Ig domains VH1, Cγ1, the hinge, Cγ2, and Cγ3 are also labeled above the sequential numbering. Polymorphisms have been observed at a number of Fc positions, including but not limited to Kabat 270, 272, 312, 315, 356, and 358, and thus slight differences between the presented sequence and sequences in the prior art may exist.

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[039] Figure 4. Experimental library residues mapped onto the Fc/FcγRIIIb complex structure 1IIS. Fc is shown as a gray ribbon diagram, and FcγRIIIb is shown as a black ribbon. Experimental library residues are shown as black ball and sticks. The N297 carbohydrate is shown as black sticks.

[040] Figure 5. The human IgG1 Fc sequence showing positions relevant to the design of the Fc variant experimental library. The sequence includes the hinge region, domain Cγ2, and domain Cγ3. Residue numbers are according to the EU index as in Kabat. Positions relevant to the experimental library are underlined. Because of observed polymorphic mutations at a number of Fc positions, slight differences between the presented sequence and sequences in the literature may exist.

[041] Figure 6. Expression of Fc variant and wild type (WT) proteins of alemtuzumab in 293T cells. Plasmids containing alemtuzumab heavy chain genes (WT or variants) were co-transfected with plasmid containing the alemtuzumab light chain gene. Media were harvested 5 days after transfection. For each transfected sample, 10ul medium was loaded on a SDS-PAGE gel for Western analysis. The probe for Western was peroxidase-conjugated goat-anti human IgG (Jackson Immuno-Research, catalog # 109-035-088). WT: wild type alemtuzumab; 1-10: alemtuzumab variants. H and L indicate antibody heavy chain and light chain, respectively.

[042] Figure 7. Purification of alemtuzumab using protein A chromatography. WT alemtuzumab proteins was expressed in 293T cells and the media was harvested 5 days after transfection. The media were diluted 1:1 with PBS and purified with protein A (Pierce, Catalog # 20334). O: original sample before purification; FT: flow through; E: elution; C: concentrated final sample. The left picture shows a Simple Blue-stained SDS-PAGE gel, and the right shows a western blot labeled using peroxidase-conjugated goat-anti human IgG.

[043] Figure 8. Production of deglycosylated antibodies. Wild type and variants of alemtuzumab were expressed in 293T cells and purified with protein A chromatography. Antibodies were incubated with peptide-N-glycosidase (PNGase F) at 37°C for 24h. For each antibody, a mock treated sample (-PNGase F) was done in parallel. WT: wild-type alemtuzumab; #15, #16, #17, #18, #22: alemtuzumab variants F241E/F243R/V262E/V264R, F241E/F243Q/V262T/V264E, F241R/F243Q/V262T/V264R, F241E/F243Y/V262T/V264R, and I332E respectively. The faster migration of the PNGase F treated versus the mock treated samples represents the deglycosylated heavy chains.

[044] Figure 9. Alemtuzumab expressed from 293T cells binds its antigen. The antigenic CD52 peptide, fused to GST, was expressed in *E. coli* BL21 (DE3) under IPTG induction. Both uninduced and induced samples were run on a SDS-PAGE gel, and transferred to PVDF membrane. For western analysis, either alemtuzumab from Sotec (□-CD52, Sotec) (final concentration 2.5ng/ul) or

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media of transfected 293T cells (Campath, Xencor) (final alemtuzumab concentration approximately 0.1-0.2ng/ul) were used as primary antibody, and peroxidase-conjugated goat-anti human IgG was used as secondary antibody. M: pre-stained marker; U: un-induced sample for GST-CD52; I: induced sample for GST-CD52.

[045] Figure 10. Expression and purification of extracellular region of human V158 FcγRIIIa. Tagged FcγRIIIa was transfected in 293T cells, and media containing secreted FcγRIIIa were harvested 3 days later and purified using affinity chromatography. 1: media; 2: flow through; 3: wash; 4-8: serial elutions. Both simple blue-stained SDS-PAGE gel and western result are shown. For the western blot, membrane was probed with anti-GST antibody.

[046] Figure 11. Binding to human V158 FcγRIIIa by select alemtuzumab Fc variants from the experimental library as determined by the AlphaScreen™ assay, described in Example 2. In the presence of competitor antibody (Fc variant or WT alemtuzumab) a characteristic inhibition curve is observed as a decrease in luminescence signal. Phosphate buffer saline (PBS) alone was used as the negative control. These data were normalized to the maximum and minimum luminescence signal provided by the baselines at low and high concentrations of competitor antibody respectively. The curves represent the fits of the data to a one site competition model using nonlinear regression. These fits provide IC50s for each antibody, illustrated for WT and S239D by the dotted lines.

[047] Figures 12. AlphaScreen™ assay showing binding of select alemtuzumab Fc variants to human FcγRIIb. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[048] Figures 13. AlphaScreen™ assay showing binding of select alemtuzumab Fc variants to human Val158 FcγRIIIa. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[049] Figure 14. AlphaScreen™ assay measuring binding to human V158 FcγRIIIa by select Fc variants in the context of rituximab. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[050] Figure 15. AlphaScreen™ assay measuring binding to human V158 FcγRIIIa by select Fc variants in the context of trastuzumab. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

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[051] Figures 16a – 16b. AlphaScreen™ assay comparing binding of select alemtuzumab Fc variants to human V158 FcγRIIIa (Figure 16a) and human FcγRIIb (Figure 16b). The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[052] Figure 17. AlphaScreen™ assay measuring binding to human V158 FcγRIIIa by select Fc variants in the context of trastuzumab. The data were normalized, and the curves represent the fits of the data to a one site competition model.

[053] Figures 18. AlphaScreen™ assay showing binding of select alemtuzumab Fc variants to human R131 FcγRIIa. The data were normalized, and the curves represent the fits of the data to a one site competition model.

[054] Figures 19a and 19b. AlphaScreen™ assay showing binding of select alemtuzumab Fc variants to human V158 FcγRIIIa. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[055] Figure 20. AlphaScreen™ assay showing binding of aglycosylated alemtuzumab Fc variants to human V158 FcγRIIIa. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[056] Figure 21. AlphaScreen™ assay comparing human V158 FcγRIIIa binding by select alemtuzumab Fc variants in glycosylated (solid symbols, solid lines) and deglycosylated (open symbols, dotted lines). The data were normalized, and the curves represent the fits of the data to a one site competition model.

[057] Figures 22a – 22b. AlphaScreen™ assay showing binding of select alemtuzumab Fc variants to the V158 (Figure 22a) and F158 (Figure 22b) allotypes of human FcγRIIIa. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[058] Figures 23a – 23d. Figures 23a and 23b show the correlation between SPR Kd's and AlphaScreen™ IC50's from binding of select alemtuzumab Fc variants to V158 FcγRIIIa (Figure 23a)

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and F158 FcγRIIIa (Figure 23b). Figures 23c and 23d show the correlation between SPR and AlphaScreen™ fold-improvements over WT for binding of select alemtuzumab Fc variants to V158 FcγRIIIa (Figure 23c) and F158 FcγRIIIa (Figure 23d). Binding data are presented in Table 62. The lines through the data represent the linear fits of the data, and the r^2 values indicate the significance of these fits.

[059] Figures 24a – 24b. Cell-based ADCC assays of select Fc variants in the context of alemtuzumab. ADCC was measured using the DELFIA® EuTDA-based cytotoxicity assay (Perkin Elmer, MA), as described in Example 7, using DoHH-2 lymphoma target cells and 50-fold excess human PBMCs. Figure 24a is a bar graph showing the raw fluorescence data for the indicated alemtuzumab antibodies at 10 ng/ml. The PBMC bar indicates basal levels of cytotoxicity in the absence of antibody. Figure 24b shows the dose-dependence of ADCC on antibody concentration for the indicated alemtuzumab antibodies, normalized to the minimum and maximum fluorescence signal provided by the baselines at low and high concentrations of antibody respectively. The curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression.

[060] Figures 25a – 25b. Cell-based ADCC assays of select Fc variants in the context of rituximab. ADCC was measured using the DELFIA® EuTDA-based cytotoxicity assay, as described in Example 7, using WIL2-S lymphoma target cells and 50-fold excess human PBMCs. Figure 25a is a bar graph showing the raw fluorescence data for the indicated rituximab antibodies at 1 ng/ml. The PBMC bar indicates basal levels of cytotoxicity in the absence of antibody. Figure 25b shows the dose-dependence of ADCC on antibody concentration for the indicated rituximab antibodies, normalized to the minimum and maximum fluorescence signal provided by the baselines at low and high concentrations of antibody respectively. The curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression.

[061] Figures 26a – 26c. Cell-based ADCC assays of select Fc variants in the context of trastuzumab. ADCC was measured using the DELFIA® EuTDA-based cytotoxicity assay, as described in Example 7, using BT474 and Sk-Br-3 breast carcinoma target cells and 50-fold excess human PBMCs. Figure 26a is a bar graph showing the raw fluorescence data for the indicated trastuzumab antibodies at 1 ng/ml. The PBMC bar indicates basal levels of cytotoxicity in the absence of antibody. Figures 26b and 26c show the dose-dependence of ADCC on antibody concentration for the indicated trastuzumab antibodies, normalized to the minimum and maximum fluorescence signal provided by the baselines at low and high concentrations of antibody respectively. The curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression.

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[062] Figures 27a – 27b. Capacity of select Fc variants to mediate binding and activation of complement. Figure 27a shows an AlphaScreen™ assay measuring binding of select alemtuzumab Fc variants to C1q. The data were normalized to the maximum and minimum luminescence signal provided by the baselines at low and high concentrations of competitor antibody respectively. The curves represent the fits of the data to a one site competition model using nonlinear regression. Figure 27b shows a cell-based assay measuring capacity of select rituximab Fc variants to mediate CDC. CDC assays were performed using Amar Blue to monitor lysis of Fc variant and WT rituximab - opsonized WIL2-S lymphoma cells by human serum complement (Quidel, San Diego, CA). The dose-dependence on antibody concentration of complement-mediated lysis is shown for the indicated rituximab antibodies, normalized to the minimum and maximum fluorescence signal provided by the baselines at low and high concentrations of antibody respectively. The curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression.

[063] Figure 28. AlphaScreen™ assay measuring binding of select alemtuzumab Fc variants to bacterial protein A, as described in Example 9. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[064] Figure 29. AlphaScreen™ assay measuring binding of select alemtuzumab Fc variants to mouse FcγRIII, as described in Example 10. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[065] Figure 30. AlphaScreen™ assay measuring binding to human V158 FcγRIIIa by select trastuzumab Fc variants expressed in 293T and CHO cells, as described in Example 11. The data were normalized, and the curves represent the fits of the data to a one site competition model. PBS was used as a negative control.

[066] Figures 31a – 31c. Sequences showing improved anti-CD20 antibodies. The light and heavy chain sequences of rituximab are presented in Figure 31a and Figure 31b respectively, and are taken from translated Sequence 3 of US 5,736,137. Relevant positions in Figure 31b are bolded, including S239, V240, V264I, N297, S298, A330, and I332. Figure 31c shows the improved anti-CD20 antibody heavy chain sequences, with variable positions designated in bold as X_1, X_2, X_3, X_4, X_5, X_6, and Z_1. The table below the sequence provides possible substitutions for these positions. The improved anti-CD20 antibody sequences comprise at least one non-WT amino acid selected from the group of possible substitutions for X_1, X_2, X_3, X_4, X_5, and X_6. These improved anti-CD20 antibody sequences may also comprise a substitution Z_1. These positions are numbered according to the EU index as in Kabat, and thus do not correspond to the sequential order in the sequence.

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DETAILED DESCRIPTION OF THE INVENTION

[067] In order that the invention may be more completely understood, several definitions are set forth below. Such definitions are meant to encompass grammatical equivalents.

[068] By "ADCC" or "antibody dependent cell-mediated cytotoxicity" as used herein is meant the cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell.

[069] By "ADCP" or antibody dependent cell-mediated phagocytosis as used herein is meant the cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell.

[070] By "amino acid modification" herein is meant an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. The preferred amino acid modification herein is a substitution. **TT

[071] By "antibody" herein is meant a protein consisting of one or more polypeptides substantially encoded by all or part of the recognized immunoglobulin genes. The recognized immunoglobulin genes, for example in humans, include the kappa (□), lambda (□), and heavy chain genetic loci, which together comprise the myriad variable region genes, and the constant region genes mu (□), delta (□), gamma (γ), sigma (□), and alpha (□) which encode the IgM, IgD, IgG, IgE, and IgA isotypes respectively. Antibody herein is meant to include full length antibodies and antibody fragments, and may refer to a natural antibody from any organism, an engineered antibody, or an antibody generated recombinantly for experimental, therapeutic, or other purposes as further defined below. Thus, "antibody" includes both polyclonal and monoclonal antibody (mAb). Methods of preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, Antibodies: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1988). As outlined herein, "antibody" specifically includes Fc variants described herein, "full length" antibodies including the Fc variant fragments described herein, and Fc variant fusions to other proteins as described herein.

[072] n some embodiments, antibodies can be neutralizing or inhibitory, or stimulatory, and in preferred embodiments, as described herein, the stimulatory activity is measured by an increase in affinitiy of a variant antibody to a receptor, as compared to either the parent antibody (e.g. when a non-naturally occurring variant is used as the starting point for the computation analysis herein), or to the original wild-type antibody. Accordingly, by "neutralization," "neutralize," "neutralizing" and grammatical equivalents herein is meant to inhibit or lessen the biological effect of the antibody, in some cases by binding (e.g. competitively) to a antigen and avoiding or decreasing the biological effect of binding, or by binding that results in decreasing the biological effect of binding.

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[073] The term "antibody" include antibody fragments, as are known in the art, such as Fab, Fab', F(ab')2, Fcs or other antigen-binding subsequences of antibodies, such as, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. Particularly preferred are Fc variants as described herein. The term "antibody" further comprises polyclonal antibodies and mAbs which can be agonist or antagonist antibodies.

[074] The antibodies of the invention specifically bind to Fc receptors, as outlined herein. By "specifically bind" herein is meant that the LC antibodies have a binding constant in the range of at least 10^{-4} - 10^{-6} M^{-1}, with a preferred range being 10^{-7} - 10^{-9} M^{-1}.
In a preferred embodiment, the antibodies of the invention are humanized. Using current monoclonal antibody technology one can produce a humanized antibody to virtually any target antigen that can be identified [Stein, Trends Biotechnol. 15:88-90 (1997)]. Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fc, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992)]. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., supra; Riechmann et al., supra; and Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Additional examples of humanized murine monoclonal antibodies are also known in the art, e.g., antibodies binding human protein C [O'Connor et al., Protein Eng. 11:321-8 (1998)], interleukin 2 receptor [Queen et al., Proc. Natl. Acad. Sci., U.S.A. 86:10029-33 (1989)], and human epidermal growth factor receptor 2 [Carter et al., Proc. Natl. Acad. Sci. U.S.A. 89:4285-9 (1992)]. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has

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been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[075] In a preferred embodiment, the antibodies of the invention are based on human sequences, and are thus human sequences are used as the "base" sequences, against which other sequences, such as rat, mouse and monkey sequences. In order to establish homology to primary sequence or structure, the amino acid sequence of a precursor or parent Fc is directly compared to the human Fc sequence outlined herein. After aligning the sequences, using one or more of the homology alignment programs described herein (for example using conserved residues as between species), allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of human Fc are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues (sometimes referred to herein as "corresponding residues").

[076] Equivalent residues may also be defined by determining homology at the level of tertiary structure for an Fc fragment whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the parent or precursor (N on N, CA on CA, C on C and O on O) are within 0.13 nm and preferably 0.1 nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the Fc variant fragment.

[077] Specifically included within the definition of "antibody" are aglycosylated antibodies. By "aglycosylated antibody" as used herein is meant an antibody that lacks carbohydrate attached at position 297 of the Fc region, wherein numbering is according to the EU system as in Kabat. The aglycosylated antibody may be a deglycosylated antibody, that is an antibody for which the Fc carbohydrate has been removed, for example chemically or enzymatically. Alternatively, the aglycosylated antibody may be a nonglycosylated or unglycosylated antibody, that is an antibody that was expressed without Fc carbohydrate, for example by mutation of one or residues that encode the glycosylation pattern or by expression in an organism that does not attach carbohydrates to proteins, for example bacteria.

[078] Specifically included within the definition of "antibody" are full-length antibodies that contain an Fc variant portion. .By "full length antibody" herein is meant the structure that constitutes the natural biological form of an antibody, including variable and constant regions. For example, in most mammals, including humans and mice, the full length antibody of the IgG class is a tetramer and consists of two identical pairs of two immunoglobulin chains, each pair having one light and one heavy

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chain, each light chain comprising immunoglobulin domains V_L and C_L, and each heavy chain comprising immunoglobulin domains V_H, $C\gamma1$, $C\gamma2$, and $C\gamma3$. In some mammals, for example in camels and llamas, IgG antibodies may consist of only two heavy chains, each heavy chain comprising a variable domain attached to the Fc region. By "IgG" as used herein is meant a polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4. In mice this class comprises IgG1, IgG2a, IgG2b, IgG3.

[079] By "amino acid" and "amino acid identity" as used herein is meant one of the 20 naturally occurring amino acids or any non-natural analogues that may be present at a specific, defined position. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures, i.e. "analogs", such as peptoids (see Simon et al., PNAS USA 89(20):9367 (1992)) particularly when LC peptides are to be administered to a patient. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homophenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chain may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradation.

[080] By "computational screening method" herein is meant any method for designing one or more mutations in a protein, wherein said method utilizes a computer to evaluate the energies of the interactions of potential amino acid side chain substitutions with each other and/or with the rest of the protein. As will be appreciated by those skilled in the art, evaluation of energies, referred to as energy calculation, refers to some method of scoring one or more amino acid modifications. Said method may involve a physical or chemical energy term, or may involve knowledge-, statistical-, sequence-based energy terms, and the like. The calculations that compose a computational screening method are herein referred to as "computational screening calculations".

[081] By "effector function" as used herein is meant a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Effector functions include but are not limited to ADCC, ADCP, and CDC. By "effector cell" as used herein is meant a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. Effector cells include but are not limited to monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and $\gamma\gamma$ T cells, and may be from any organism including but not limited to humans, mice,

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rats, rabbits, and monkeys. By "library" herein is meant a set of Fc variants in any form, including but not limited to a list of nucleic acid or amino acid sequences, a list of nucleic acid or amino acid substitutions at variable positions, a physical library comprising nucleic acids that encode the library sequences, or a physical library comprising the Fc variant proteins, either in purified or unpurified form.

[082] By "Fc", "Fc region", FC polypeptide", etc. as used herein is meant an antibody as defined herein that includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, as illustrated in Figure 1, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cγ2 and Cγ3) and the hinge between Cgamma1 (Cγ1) and Cgamma2 (Cγ2). Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as in Kabat. Fc may refer to this region in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion. An Fc may be an antibody, Fc fusion, or an protein or protein domain that comprises Fc. Particularly preferred are Fc variants, which are non-naturally occurring variants of an Fc.

[083] By "Fc fusion" as used herein is meant a protein wherein one or more polypeptides is operably linked to Fc. Fc fusion is herein meant to be synonymous with the terms "immunoadhesin", "Ig fusion", "Ig chimera", and "receptor globulin" (sometimes with dashes) as used in the prior art (Chamow *et al.*, 1996, *Trends Biotechnol* 14:52-60; Ashkenazi *et al.*, 1997, *Curr Opin Immunol* 9:195-200). An Fc fusion combines the Fc region of an immunoglobulin with a fusion partner, which in general can be any protein, including, but not limited to, the target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme, or some other protein or protein domain. The role of the non-Fc part of an Fc fusion is to mediate target binding, and thus it is functionally analogous to the variable regions of an antibody.

[084] By "Fc gamma receptor" or "FcγR" as used herein is meant any member of the family of proteins that bind the IgG antibody Fc region and are substantially encoded by the FcγR genes. In humans this family includes but is not limited to FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2) (Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65), as well as any undiscovered human FcγRs or FcγR isoforms or allotypes. An FcγR may be from any organism, including but not limited to humans,

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mice, rats, rabbits, and monkeys. Mouse FcγRs include but are not limited to FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIII-2 (CD16-2), as well as any undiscovered mouse FcγRs or FcγR isoforms or allotypes.

[085] By "Fc ligand" as used herein is meant a molecule, preferably a polypeptide, from any organism that binds to the Fc region of an antibody to form an Fc-ligand complex. Fc ligands include but are not limited to FcγRs, FcγRs, FcγRs, FcRn, C1q, C3, mannan binding lectin, mannose receptor, *staphylococcal* protein A, *streptococcal* protein G, and viral FcγR. Fc ligands may include undiscovered molecules that bind Fc

[086] By "IgG" as used herein is meant a polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4. In mice this class comprises IgG1, IgG2a, IgG2b, IgG3. By "immunoglobulin (Ig)" herein is meant a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. Immunoglobulins include but are not limited to antibodies. Immunoglobulins may have a number of structural forms, including but not limited to full length antibodies, antibody fragments, and individual immunoglobulin domains. By "immunoglobulin (Ig) domain" herein is meant a region of an immunoglobulin that exists as a distinct structural entity as ascertained by one skilled in the art of protein structure. Ig domains typically have a characteristic □-sandwich folding topology. The known Ig domains in the IgG class of antibodies are V_H, Cγ1, Cγ2, Cγ3, V_L, and C_L.

[087] By "parent polypeptide" or "precursor polypeptide" (including Fc parent or precursors) as used herein is meant a polypeptide that is subsequently modified to generate a variant. Said parent polypeptide may be a naturally occurring polypeptide, or a variant or engineered version of a naturally occurring polypeptide. Parent polypeptide may refer to the polypeptide itself, compositions that comprise the parent polypeptide, or the amino acid sequence that encodes it. Accordingly, by "parent Fc polypeptide" as used herein is meant an unmodified Fc polypeptide that is modified to generate a variant, and by "parent antibody" as used herein is meant an unmodified antibody that is modified to generate a variant antibody.

[088] As outlined above, certain positions of the Fc molecule can be altered. By "position" as used herein is meant a location in the sequence of a protein. Positions may be numbered sequentially, or according to an established format, for example the EU index as in Kabat. For example, position 297 is a position in the human antibody IgG1. Corresponding positions are determined as outlined above, generally through alignment with other parent sequences.

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[089] By "residue" as used herein is meant a position in a protein and its associated amino acid identity. For example, Daragine 297 (also referred to as N297, also referred to as N297) is a residue in the human antibody IgG1.

[090] By "target antigen" as used herein is meant the molecule that is bound specifically by the variable region of a given antibody. A target antigen may be a protein, carbohydrate, lipid, or other chemical compound.

[091] By "target cell" as used herein is meant a cell that expresses a target antigen.

[092] By "variable region" as used herein is meant the region of an immunoglobulin that comprises one or more Ig domains substantially encoded by any of the V□, V□, and/or V_H genes that make up the kappa, lambda, and heavy chain immunoglobulin genetic loci respectively.

[093] By "variant polypeptide" as used herein is meant a polypeptide sequence that differs from that of a parent polypeptide sequence by virtue of at least one amino acid modification. Variant polypeptide may refer to the polypeptide itself, a composition comprising the polypeptide, or the amino sequence that encodes it. Preferably, the variant polypeptide has at least one amino acid modification compared to the parent polypeptide, e.g. from about one to about ten amino acid modifications, and preferably from about one to about five amino acid modifications compared to the parent. The variant polypeptide sequence herein will preferably possess at least about 80% homology with a parent polypeptide sequence, and most preferably at least about 90% homology, more preferably at least about 95% homology. Accordingly, by "Fc variant" as used herein is meant an Fc sequence that differs from that of a parent Fc sequence by virtue of at least one amino acid modification. An Fc variant may only encompass an Fc region, or may exist in the context of an antibody, Fc fusion, or other polypeptide that is substantially encoded by Fc. Fc variant may refer to the Fc polypeptide itself, compositions comprising the Fc variant polypeptide, or the amino acid sequence that encodes it.

[094] For all positions discussed in the present invention, numbering of an immunoglobulin heavy chain is according to the EU index (Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th Ed., United States Public Health Svice, National Institutes of Health, Bethesda). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

[095] The Fc variants of the present invention may be optimized for a variety of properties. Properties that may be optimized include but are not limited to enhanced or reduced affinity for an

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FcγR. In a preferred embodiment, the Fc variants of the present invention are optimized to possess enhanced affinity for a human activating FcγR, preferably FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb, most preferably FcγRIIIa. In an alternately preferred embodiment, the Fc variants are optimized to possess reduced affinity for the human inhibitory receptor FcγRIIb. These preferred embodiments are anticipated to provide antibodies and Fc fusions with enhanced therapeutic properties in humans, for example enhanced effector function and greater anti-cancer potency. In an alternate embodiment, the Fc variants of the present invention are optimized to have reduced or ablated affinity for a human FcγR, including but not limited to FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb. These embodiments are anticipated to provide antibodies and Fc fusions with enhanced therapeutic properties in humans, for example reduced effector function and reduced toxicity. Preferred embodiments comprise optimization of Fc binding to a human FcγR, however in alternate embodiments the Fc variants of the present invention possess enhanced or reduced affinity for FcγRs from nonhuman organisms, including but not limited to mice, rats, rabbits, and monkeys. Fc variants that are optimized for binding to a nonhuman FcγR may find use in experimentation. For example, mouse models are available for a variety of diseases that enable testing of properties such as efficacy, toxicity, and pharmacokinetics for a given drug candidate. As is known in the art, cancer cells can be grafted or injected into mice to mimic a human cancer, a process referred to as xenografting. Testing of antibodies or Fc fusions that comprise Fc variants that are optimized for one or more mouse FcγRs, may provide valuable information with regard to the efficacy of the antibody or Fc fusion, its mechanism of action, and the like. The Fc variants of the present invention may also be optimized for enhanced functionality and/or solution properties in aglycosylated form. In a preferred embodiment, the aglycosylated Fc variants of the present invention bind an Fc ligand with greater affinity than the aglycosylated form of the parent Fc polypeptide. Said Fc ligands include but are not limited to FcγRs, C1q, FcRn, and proteins A and G, and may be from any source including but not limited to human, mouse, rat, rabbit, or monkey, preferably human. In an alternately preferred embodiment, the Fc variants are optimized to be more stable and/or more soluble than the aglycosylated form of the parent Fc polypeptide. An Fc variant that is engineered or predicted to display any of the aforementioned optimized properties is herein referred to as an "optimized Fc variant".

[096] The Fc variants of the present invention may be derived from parent Fc polypeptides that are themselves from a wide range of sources. The parent Fc polypeptide may be substantially encoded by one or more Fc genes from any organism, including but not limited to humans, mice, rats, rabbits, camels, llamas, dromedaries, monkeys, preferably mammals and most preferably humans and mice. In a preferred embodiment, the parent Fc polypeptide composes an antibody, referred to as the parent antibody. The parent antibody may be fully human, obtained for example using transgenic mice (Bruggemann *et al.*, 1997, *Curr Opin Biotechnol* 8:455-458) or human antibody libraries coupled with selection methods (Griffiths *et al.*, 1998, *Curr Opin Biotechnol* 9:102-108). The parent antibody need not be naturally occurring. For example, the parent antibody may be an engineered antibody,

including but not limited to chimeric antibodies and humanized antibodies (Clark, 2000, *Immunol Today* 21:397-402). The parent antibody may be an engineered variant of an antibody that is substantially encoded by one or more natural antibody genes. In one embodiment, the parent antibody has been affinity matured, as is known in the art. Alternatively, the antibody has been modified in some other way, for example as described in USSN 10/339788, filed on March 3, 2003.

[097] The Fc variants of the present invention may be substantially encoded by immunoglobulin genes belonging to any of the antibody classes. In a preferred embodiment, the Fc variants of the present invention find use in antibodies or Fc fusions that comprise sequences belonging to the IgG class of antibodies, including IgG1, IgG2, IgG3, or IgG4. In an alternate embodiment the Fc variants of the present invention find use in antibodies or Fc fusions that comprise sequences belonging to the IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG, or IgM classes of antibodies. The Fc variants of the present invention may comprise more than one protein chain. That is, the present invention may find use in an antibody or Fc fusion that is a monomer or an oligomer, including a homo- or hetero-oligomer.

[098] The Fc variants of the present invention may be combined with other Fc modifications, including but not limited to modifications that alter effector function. Such combination may provide additive, synergistic, or novel properties in antibodies or Fc fusions. In one embodiment, the Fc variants of the present invention may be combined with other known Fc variants (Duncan *et al.*, 1988, *Nature* 332:563-564; Lund *et al.*, 1991, *J Immunol* 147:2657-2662; Lund *et al.*, 1992, *Mol Immunol* 29:53-59; Alegre *et al.*, 1994, *Transplantation* 57:1537-1543; Hutchins *et al.*, 1995, *Proc Natl Acad Sci U S A* 92:11980-11984; Jefferis *et al.*, 1995, *Immunol Lett* 44:111-117; Lund *et al.*, 1995, *Faseb J* 9:115-119; Jefferis *et al.*, 1996, *Immunol Lett* 54:101-104; Lund *et al.*, 1996, *J Immunol* 157:4963-4969; Armour *et al.*, 1999, *Eur J Immunol* 29:2613-2624; Idusogie *et al.*, 2000, *J Immunol* 164:4178-4184; Reddy *et al.*, 2000, *J Immunol* 164:1925-1933; Xu *et al.*, 2000, *Cell Immunol* 200:16-26; Idusogie *et al.*, 2001, *J Immunol* 166:2571-2575; Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604; Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65; Presta *et al.*, 2002, *Biochem Soc Trans* 30:487-490) (US 5,624,821; US 5,885,573; US 6,194,551; PCT WO 00/42072; PCT WO 99/58572). In an alternate embodiment, the Fc variants of the present invention are incorporated into an antibody or Fc fusion that comprises one or more engineered glycoforms. By "engineered glycoform" as used herein is meant a carbohydrate composition that is covalently attached to an Fc polypeptide, wherein said carbohydrate composition differs chemically from that of a parent Fc polypeptide. Engineered glycoforms may be useful for a variety of purposes, including but not limited to enhancing or reducing effector function. Engineered glycoforms may be generated by any method, for example by using engineered or variant expression strains, by co-expression with one or more enzymes, for example □1–4- N-acetylglucosaminyltransferase III (GnTIII), by expressing an Fc polypeptide in various organisms or cell lines from various organisms, or by modifying carbohydrate(s) after the Fc polypeptide has been expressed. Methods for generating engineered glycoforms are known in the

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art, and include but are not limited to (Umaña *et al.*, 1999, *Nat Biotechnol* 17:176-180; Davies *et al.*, 2001, *Biotechnol Bioeng* 74:288-294; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740; Shinkawa *et al.*, 2003, *J Biol Chem* 278:3466-3473) US 6,602,684; USSN 10/277,370; USSN 10/113,929; PCT WO 00/61739A1; PCT WO 01/29246A1; PCT WO 02/31140A1; PCT WO 02/30954A1; Potelligent™ technology (Biowa, Inc., Princeton, N.J.); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG, Zürich, Switzerland)). Engineered glycoform typically refers to the different carbohydrate or oligosaccharide; thus an Fc polypeptide, for example an antibody or Fc fusion, may comprise an engineered glycoform. Alternatively, engineered glycoform may refer to the Fc polypeptide that comprises the different carbohydrate or oligosaccharide. Thus combinations of the Fc variants of the present invention with other Fc modifications, as well as undiscovered Fc modifications, are contemplated with the goal of generating novel antibodies or Fc fusions with optimized properties.

[099] The Fc variants of the present invention may find use in an antibody. By "antibody of the present invention" as used herein is meant an antibody that comprises an Fc variant of the present invention. The present invention may, in fact, find use in any protein that comprises Fc, and thus application of the Fc variants of the present invention is not limited to antibodies. The Fc variants of the present invention may find use in an Fc fusion. By "Fc fusion of the present invention" as used herein refers to an Fc fusion that comprises an Fc variant of the present invention. Fc fusions may comprise an Fc variant of the present invention operably linked to a cytokine, soluble receptor domain, adhesion molecule, ligand, enzyme, peptide, or other protein or protein domain, and include but are not limited to Fc fusions described in US 5,843,725; US 6,018,026; US 6,291,212; US 6,291,646; US 6,300,099; US 6,323,323; PCT WO 00/24782; and in (Chamow *et al.*, 1996, *Trends Biotechnol* 14:52-60; Ashkenazi *et al.*, 1997, *Curr Opin Immunol* 9:195-200).

[100] Virtually any antigen may be targeted by the antibodies and fusions of the present invention, including but are not limited to the following list of proteins, subunits, domains, motifs, and epitopes belonging to the following list of proteins: CD2; CD3, CD3E, CD4, CD11, CD11a, CD14, CD16, CD18, CD19, CD20, CD22, CD23, CD25, CD28, CD29, CD30, CD32, CD33 (p67 protein), CD38, CD40, CD40L, CD52, CD54, CD56, CD80, CD147, GD3, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-6R, IL-8, IL-12, IL-15, IL-18, IL-23, interferon alpha, interferon beta, interferon gamma; TNF-alpha, TNFbeta2, TNFc, TNFalphabeta, TNF-RI, TNF-RII, FasL, CD27L, CD30L, 4-1BBL, TRAIL, RANKL, TWEAK, APRIL, BAFF, LIGHT, VEGI, OX40L, TRAIL Receptor-1, A1 Adenosine Receptor, Lymphotoxin Beta Receptor, TACI, BAFF-R, EPO; LFA-3, ICAM-1, ICAM-3, EpCAM, integrin beta1, integrin beta2, integrin alpha4/beta7, integrin alpha2, integrin alpha3, integrin alpha4, integrin alpha5, integrin alpha6, integrin alphav, alphaVbeta3 integrin, FGFR-3, Keratinocyte Growth Factor, VLA-1, VLA-4, L-selectin, anti-Id, E-selectin, HLA, HLA-DR, CTLA-4, T cell receptor, B7-1, B7-2, VNRintegrin, TGFbeta1, TGFbeta2, eotaxin1, BLyS (B-lymphocyte Stimulator), complement C5, IgE, factor VII, CD64, CBL, NCA 90, EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), Tissue

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Factor, VEGF, VEGFR, endothelin receptor, VLA-4, Hapten NP-cap or NIP-cap, T cell receptor alpha/beta, E-selectin, digoxin, placental alkaline phosphatase (PLAP) and testicular PLAP-like alkaline phosphatase, transferrin receptor, Carcinoembryonic antigen (CEA), CEACAM5, HMFG PEM, mucin MUC1, MUC18, Heparanase I, human cardiac myosin, tumor-associated glycoprotein-72 (TAG-72), tumor-associated antigen CA 125, Prostate specific membrane antigen (PSMA), High molecular weight melanoma-associated antigen (HMW-MAA), carcinoma-associated antigen, Gcoprotein IIb/IIIa (GPIIb/IIIa), tumor-associated antigen expressing Lewis Y related carbohydrate, human cytomegalovirus (HCMV) gH envelope glycoprotein, HIV gp120, HCMV, respiratory syncital virus RSV F, RSVF Fgp, VNRintegrin, IL-8, cytokeratin tumor-associated antigen, Hep B gp120, CMV, gpIIbIIIa, HIV IIIB gp120 V3 loop, respiratory syncytial virus (RSV) Fgp, Herpes simplex virus (HSV) gD glycoprotein, HSV gB glycoprotein, HCMV gB envelope glycoprotein, and *Clostridium perfringens* toxin.

[101] One skilled in the art will appreciate that the aforementioned list of targets refers not only to specific proteins and biomolecules, but the biochemical pathway or pathways that comprise them. For example, reference to CTLA-4 as a target antigen implies that the ligands and receptors that make up the T cell co-stimulatory pathway, including CTLA-4, B7-1, B7-2, CD28, and any other undiscovered ligands or receptors that bind these proteins, are also targets. Thus target as used herein refers not only to a specific biomolecule, but the set of proteins that interact with said target and the members of the biochemical pathway to which said target belongs. One skilled in the art will further appreciate that any of the aforementioned target antigens, the ligands or receptors that bind them, or other members of their corresponding biochemical pathway, may be operably linked to the Fc variants of the present invention in order to generate an Fc fusion. Thus for example, an Fc fusion that targets EGFR could be constructed by operably linking an Fc variant to EGF, TGF□, or any other ligand, discovered or undiscovered, that binds EGFR. Accordingly, an Fc variant of the present invention could be operably linked to EGFR in order to generate an Fc fusion that binds EGF, TGF□, or any other ligand, discovered or undiscovered, that binds EGFR. Thus virtually any polypeptide, whether a ligand, receptor, or some other protein or protein domain, including but not limited to the aforementioned targets and the proteins that compose their corresponding biochemical pathways, may be operably linked to the Fc variants of the present invention to develop an Fc fusion.

[102] A number of antibodies and Fc fusions that are approved for use, in clinical trials, or in development may benefit from the Fc variants of the present invention. Said antibodies and Fc fusions are herein referred to as "clinical products and candidates". Thus in a preferred embodiment, the Fc variants of the present invention may find use in a range of clinical products and candidates. For example, a number of antibodies that target CD20 may benefit from the Fc variants of the present invention. For example the Fc variants of the present invention may find use in an antibody that is substantially similar to rituximab (Rituxan®, IDEC/Genentech/Roche) (see for example US 5,736,137), a chimeric anti-CD20 antibody approved to treat Non-Hodgkin's lymphoma; HuMax-

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CD20, an anti-CD20 currently being developed by Genmab, an anti-CD20 antibody described in US 5,500,362, AME-133 (Applied Molecular Evolution), hA20 (Immunomedics, Inc.), and HumaLYM (Intracel). A number of antibodies that target members of the family of epidermal growth factor receptors, including EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), may benefit from the Fc variants of the present invention. For example the Fc variants of the present invention may find use in an antibody that is substantially similar to trastuzumab (Herceptin®, Genentech) (see for example US 5,677,171), a humanized anti-Her2/neu antibody approved to treat breast cancer; pertuzumab (rhuMab-2C4, Omnitarg™), currently being developed by Genentech; an anti-Her2 antibody described in US 4,753,894; cetuximab (Erbitux®, Imclone) (US 4,943,533; PCT WO 96/40210), a chimeric anti-EGFR antibody in clinical trials for a variety of cancers; ABX-EGF (US 6,235,883), currently being developed by Abgenix/Immunex/Amgen; HuMax-EGFr (USSN 10/172,317), currently being developed by Genmab; 425, EMD55900, EMD62000, and EMD72000 (Merck KGaA) (US 5558864; Murthy et al. 1987, *Arch Biochem Biophys.* 252(2):549-60; Rodeck et al., 1987, *J Cell Biochem.* 35(4):315-20; Kettleborough et al., 1991, *Protein Eng.* 4(7):773-83); ICR62 (Institute of Cancer Research) (PCT WO 95/20045; Modjtahedi et al., 1993, *J. Cell Biophys.* 1993, 22(1-3):129-46; Modjtahedi et al., 1993, *Br J Cancer.* 1993, 67(2):247-53; Modjtahedi et al, 1996, *Br J Cancer*, 73(2):228-35; Modjtahedi et al, 2003, *Int J Cancer,* 105(2):273-80); TheraCIM hR3 (YM Biosciences, Canada and Centro de Immunologia Molecular, Cuba (US 5,891,996; US 6, 506,883; Mateo et al, 1997, *Immunotechnology*, 3(1):71-81); mAb-806 (Ludwig Institue for Cancer Research, Memorial Sloan-Kettering) (Jungbluth et al. 2003, *Proc Natl Acad Sci U S A.* 100(2):639-44); KSB-102 (KS Biomedix); MR1-1 (IVAX, National Cancer Institute) (PCT WO 0162931A2); and SC100 (Scancell) (PCT WO 01/88138). In another preferred embodiment, the Fc variants of the present invention may find use in alemtuzumab (Campath®, Millenium), a humanized monoclonal antibody currently approved for treatment of B-cell chronic lymphocytic leukemia. The Fc variants of the present invention may find use in a variety of antibodies or Fc fusions that are substantially similar to other clinical products and candidates, including but not limited to muromonab-CD3 (Orthoclone OKT3®), an anti-CD3 antibody developed by Ortho Biotech/Johnson & Johnson, ibritumomab tiuxetan (Zevalin®), an anti-CD20 antibody developed by IDEC/Schering AG, gemtuzumab ozogamicin (Mylotarg®), an anti-CD33 (p67 protein) antibody developed by Celltech/Wyeth, alefacept (Amevive®), an anti-LFA-3 Fc fusion developed by Biogen), abciximab (ReoPro®), developed by Centocor/Lilly, basiliximab (Simulect®), developed by Novartis, palivizumab (Synagis®), developed by MedImmune, infliximab (Remicade®), an anti-TNFalpha antibody developed by Centocor, adalimumab (Humira®, an anti-TNFalpha antibody developed by Abbott, Humicade™, an anti-TNFalpha antibody developed by Celltech, etanercept (Enbrel®), an anti-TNFalpha Fc fusion developed by Immunex/Amgen, ABX-CBL, an anti-CD147 antibody being developed by Abgenix, ABX-IL8, an anti-IL8 antibody being developed by Abgenix, ABX-MA1, an anti-MUC18 antibody being developed by Abgenix, Pemtumomab (R1549, ^{90}Y-muHMFG1), an anti-MUC1 In development by Antisoma, Therex (R1550), an anti-MUC1 antibody being developed by Antisoma, AngioMab (AS1405), being developed by Antisoma, HuBC-1, being developed by Antisoma, Thioplatin (AS1407) being developed by Antisoma, Antegren® (natalizumab), an anti-alpha-4-beta-1 (VLA-4) and alpha-4-

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beta-7 antibody being developed by Biogen, VLA-1 mAb, an anti-VLA-1 integrin antibody being developed by Biogen, LTBR mAb, an anti-lymphotoxin beta receptor (LTBR) antibody being developed by Biogen, CAT-152, an anti-TGF□2 antibody being developed by Cambridge Antibody Technology, J695, an anti-IL-12 antibody being developed by Cambridge Antibody Technology and Abbott, CAT-192, an anti-TGF□1 antibody being developed by Cambridge Antibody Technology and Genzyme, CAT-213, an anti-Eotaxin1 antibody being developed by Cambridge Antibody Technology, LymphoStat-B™ an anti-Blys antibody being developed by Cambridge Antibody Technology and Human Genome Sciences Inc., TRAIL-R1mAb, an anti-TRAIL-R1 antibody being developed by Cambridge Antibody Technology and Human Genome Sciences, Inc., Avastin™ (bevacizumab, rhuMAb-VEGF), an anti-VEGF antibody being developed by Genentech, an anti-HER receptor family antibody being developed by Genentech, Anti-Tissue Factor (ATF), an anti-Tissue Factor antibody being developed by Genentech, Xolair™ (Omalizumab), an anti-IgE antibody being developed by Genentech, Raptiva™ (Efalizumab), an anti-CD11a antibody being developed by Genentech and Xoma, MLN-02 Antibody (formerly LDP-02), being developed by Genentech and Millenium Pharmaceuticals, HuMax CD4, an anti-CD4 antibody being developed by Genmab, HuMax-IL15, an anti-IL15 antibody being developed by Genmab and Amgen, HuMax-Inflam, being developed by Genmab and Medarex, HuMax-Cancer, an anti-Heparanase I antibody being developed by Genmab and Medarex and Oxford GcoSciences, HuMax-Lymphoma, being developed by Genmab and Amgen, HuMax-TAC, being developed by Genmab, IDEC-131, and anti-CD40L antibody being developed by IDEC Pharmaceuticals, IDEC-151 (Clenoliximab), an anti-CD4 antibody being developed by IDEC Pharmaceuticals, IDEC-114, an anti-CD80 antibody being developed by IDEC Pharmaceuticals, IDEC-152, an anti-CD23 being developed by IDEC Pharmaceuticals, anti-macrophage migration factor (MIF) antibodies being developed by IDEC Pharmaceuticals, BEC2, an anti-idiotypic antibody being developed by Imclone, IMC-1C11, an anti-KDR antibody being developed by Imclone, DC101, an anti-flk-1 antibody being developed by Imclone, anti-VE cadherin antibodies being developed by Imclone, CEA-Cide™ (labetuzumab), an anti-carcinoembryonic antigen (CEA) antibody being developed by Immunomedics, LymphoCide™ (Epratuzumab), an anti-CD22 antibody being developed by Immunomedics, AFP-Cide, being developed by Immunomedics, MyelomaCide, being developed by Immunomedics, LkoCide, being developed by Immunomedics, ProstaCide, being developed by Immunomedics, MDX-010, an anti-CTLA4 antibody being developed by Medarex, MDX-060, an anti-CD30 antibody being developed by Medarex, MDX-070 being developed by Medarex, MDX-018 being developed by Medarex, Osidem™ (IDM-1), and anti-Her2 antibody being developed by Medarex and Immuno-Designed Molecules, HuMax™-CD4, an anti-CD4 antibody being developed by Medarex and Genmab, HuMax-IL15, an anti-IL15 antibody being developed by Medarex and Genmab, CNTO 148, an anti-TNF□ antibody being developed by Medarex and Centocor/J&J, CNTO 1275, an anti-cytokine antibody being developed by Centocor/J&J, MOR101 and MOR102, anti-intercellular adhesion molecule-1 (ICAM-1) (CD54) antibodies being developed by MorphoSys, MOR201, an anti-fibroblast growth factor receptor 3 (FGFR-3) antibody being developed by MorphoSys, Nuvion® (visilizumab), an anti-CD3 antibody being developed by Protein Design Labs, HuZAF™, an anti-gamma interferon antibody being developed by Protein Design Labs, Anti-□5□1

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Integrin, being developed by Protein Design Labs, anti-IL-12, being developed by Protein Design Labs, ING-1, an anti-Ep-CAM antibody being developed by Xoma, and MLN01, an anti-Beta2 integrin antibody being developed by Xoma.

[103] Application of the Fc variants to the aforementioned antibody and Fc fusion clinical products and candidates is not meant to be constrained to their precise composition. The Fc variants of the present invention may be incorporated into the aforementioned clinical candidates and products, or into antibodies and Fc fusions that are substantially similar to them. The Fc variants of the present invention may be incorporated into versions of the aforementioned clinical candidates and products that are humanized, affinity matured, engineered, or modified in some other way. Furthermore, the entire polypeptide of the aforementioned clinical products and candidates need not be used to construct a new antibody or Fc fusion that incorporates the Fc variants of the present invention; for example only the variable region of a clinical product or candidate antibody, a substantially similar variable region, or a humanized, affinity matured, engineered, or modified version of the variable region may be used. In another embodiment, the Fc variants of the present invention may find use in an antibody or Fc fusion that binds to the same epitope, antigen, ligand, or receptor as one of the aforementioned clinical products and candidates.

[104] The Fc variants of the present invention may find use in a wide range of antibody and Fc fusion products. In one embodiment the antibody or Fc fusion of the present invention is a therapeutic, a diagnostic, or a research reagent, preferably a therapeutic. Alternatively, the antibodies and Fc fusions of the present invention may be used for agricultural or industrial uses. In an alternate embodiment, the Fc variants of the present invention compose a library that may be screened experimentally. This library may be a list of nucleic acid or amino acid sequences, or may be a physical composition of nucleic acids or polypeptides that encode the library sequences. The Fc variant may find use in an antibody composition that is monoclonal or polyclonal. In a preferred embodiment, the antibodies and Fc fusions of the present invention are used to kill target cells that bear the target antigen, for example cancer cells. In an alternate embodiment, the antibodies and Fc fusions of the present invention are used to block, antagonize, or agonize the target antigen, for example for antagonizing a cytokine or cytokine receptor. In an alternately preferred embodiment, the antibodies and Fc fusions of the present invention are used to block, antagonize, or agonize the target antigen and kill the target cells that bear the target antigen.

[105] The Fc variants of the present invention may be used for various therapeutic purposes. In a preferred embodiment, the Fc variant proteins are administered to a patient to treat an antibody-related disorder. A "patient" for the purposes of the present invention includes both humans and other animals, preferably mammals and most preferably humans. Thus the antibodies and Fc fusions of the present invention have both human therapy and veterinary applications. In the preferred embodiment

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the patient is a mammal, and in the most preferred embodiment the patient is human. The term "treatment" in the present invention is meant to include therapeutic treatment, as well as prophylactic, or suppressive measures for a disease or disorder. Thus, for example, successful administration of an antibody or Fc fusion prior to onset of the disease results in treatment of the disease. As another example, successful administration of an optimized antibody or Fc fusion after clinical manifestation of the disease to combat the symptoms of the disease comprises treatment of the disease. "Treatment" also encompasses administration of an optimized antibody or Fc fusion protein after the appearance of the disease in order to eradicate the disease. Successful administration of an agent after onset and after clinical symptoms have developed, with possible abatement of clinical symptoms and perhaps amelioration of the disease, comprises treatment of the disease. Those "in need of treatment" include mammals already having the disease or disorder, as well as those prone to having the disease or disorder, including those in which the disease or disorder is to be prevented. By "antibody related disorder" or "antibody responsive disorder" or "condition" or "disease" herein are meant a disorder that may be ameliorated by the administration of a pharmaceutical composition comprising an antibody or Fc fusion of the present invention. Antibody related disorders include but are not limited to autoimmune diseases, immunological diseases, infectious diseases, inflammatory diseases, neurological diseases, and oncological and neoplastic diseases including cancer. By "cancer" and "cancerous" herein refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma (including liposarcoma), neuroendocrine tumors, mesothelioma, schwanoma, meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer. Furthermore, the Fc variants of the present invention may be used to treat conditions including but not limited to congestive heart failure (CHF), vasculitis, rosecea, acne, eczema, myocarditis and other conditions of the myocardium, systemic lupus erythematosus, diabetes, spondylopathies, synovial fibroblasts, and bone marrow stroma; bone loss; Paget's disease, osteoclastoma; multiple myeloma; breast cancer; disuse osteopenia; malnutrition, periodontal disease, Gaucher's disease, Langerhans' cell histiocytosis, spinal cord injury, acute septic arthritis, osteomalacia, Cushing's syndrome, monoostotic fibrous dysplasia, polyostotic fibrous dysplasia, periodontal reconstruction, and bone fractures; sarcoidosis; multiple myeloma; osteolytic bone cancers, breast cancer, lung cancer, kidney cancer and rectal cancer; bone metastasis, bone pain management, and humoral malignant hypercalcemia, ankylosing spondylitisa and other spondyloarthropathies; transplantation rejection, viral infections, hematologic neoplasisas and

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neoplastic-like conditions for example, Hodgkin's lymphoma; non-Hodgkin's lymphomas (Burkitt's lymphoma, small lymphocytic lymphoma/chronic lymphocytic leukemia, mycosis fungoides, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, marginal zone lymphoma, hairy cell leukemia and lymphoplasmacytic leukemia), tumors of lymphocyte precursor cells, including B-cell acute lymphoblastic leukemia/lymphoma, and T-cell acute lymphoblastic leukemia/lymphoma, thymoma, tumors of the mature T and NK cells, including peripheral T-cell leukemias, adult T-cell leukemia/T-cell lymphomas and large granular lymphocytic leukemia, Langerhans cell histocytosis, myeloid neoplasias such as acute myelogenous leukemias, including AML with maturation, AML without differentiation, acute promyelocytic leukemia, acute myelomonocytic leukemia, and acute monocytic leukemias, myelodysplastic syndromes, and chronic myeloproliferative disorders, including chronic myelogenous leukemia, tumors of the central nervous system, e.g., brain tumors (glioma, neuroblastoma, astrocytoma, medulloblastoma, ependymoma, and retinoblastoma), solid tumors (nasopharyngeal cancer, basal cell carcinoma, pancreatic cancer, cancer of the bile duct, Kaposi's sarcoma, testicular cancer, uterine, vaginal or cervical cancers, ovarian cancer, primary liver cancer or endometrial cancer, and tumors of the vascular system (angiosarcoma and hemagiopericytoma), osteoporosis, hepatitis, HIV, AIDS, spondyloarthritis, rheumatoid arthritis, inflammatory bowel diseases (IBD), sepsis and septic shock, Crohn's Disease, psoriasis, schleraderma, graft versus host disease (GVHD), allogenic islet graft rejection, hematologic malignancies, such as multiple myeloma (MM), myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), inflammation associated with tumors, peripheral nerve injury or demyelinating diseases.

[106] In one embodiment, an antibody or Fc fusion of the present invention is administered to a patient having a disease involving inappropriate expression of a protein. Within the scope of the present invention this is meant to include diseases and disorders characterized by aberrant proteins, due for example to alterations in the amount of a protein present, the presence of a mutant protein, or both. An overabundance may be due to any cause, including but not limited to overexpression at the molecular level, prolonged or accumulated appearance at the site of action, or increased activity of a protein relative to normal. Included within this definition are diseases and disorders characterized by a reduction of a protein. This reduction may be due to any cause, including but not limited to reduced expression at the molecular level, shortened or reduced appearance at the site of action, mutant forms of a protein, or decreased activity of a protein relative to normal. Such an overabundance or reduction of a protein can be measured relative to normal expression, appearance, or activity of a protein, and said measurement may play an important role in the development and/or clinical testing of the antibodies and Fc fusions of the present invention.

[107] In one embodiment, an antibody or Fc fusion of the present invention is the only therapeutically active agent administered to a patient. Alternatively, the antibody or Fc fusion of the present invention is administered in combination with one or more other therapeutic agents, including but not limited to cytotoxic agents, chemotherapeutic agents, cytokines, growth inhibitory agents, anti-

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hormonal agents, kinase inhibitors, anti-angiogenic agents, cardioprotectants, or other therapeutic agents. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. The skilled medical practitioner can determine empirically the appropriate dose or doses of other therapeutic agents useful herein. The antibodies and Fc fusions of the present invention may be administered concomitantly with one or more other therapeutic regimens. For example, an antibody or Fc fusion of the present invention may be administered to the patient along with chemotherapy, radiation therapy, or both chemotherapy and radiation therapy. In one embodiment, the antibody or Fc fusion of the present invention may be administered in conjunction with one or more antibodies or Fc fusions, which may or may not comprise an Fc variant of the present invention.

[108] In one embodiment, the antibodies and Fc fusions of the present invention are administered with a chemotherapeutic agent. By "chemotherapeutic agent" as used herein is meant a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include but are not limited to alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb

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Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhne-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; thymidylate synthase inhibitor (such as Tomudex); cox-2 inhibitors, such as celicoxib (CELEBREX®) or MK-0966 (VIOXX®); and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[109] A chemotherapeutic or other cytotoxic agent may be administered as a prodrug. By "prodrug" as used herein is meant a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, for example Wilman, 1986, Biochemical Society Transactions, 615th Meeting Belfast, 14:375-382; and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt *et al.*, (ed.): 247-267, Humana Press, 1985. The prodrugs that may find use with the present invention include but are not limited to phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use with the antibodies and Fc fusions of the present invention include but are not limited to any of the aforementioned chemotherapeutic agents.

[110] The antibodies and Fc fusions of the present invention may be combined with other therapeutic regimens. For example, in one embodiment, the patient to be treated with the antibody or Fc fusion may also receive radiation therapy. Radiation therapy can be administered according to protocols commonly employed in the art and known to the skilled artisan. Such therapy includes but is not limited to cesium, iridium, iodine, or cobalt radiation. The radiation therapy may be whole body irradiation, or may be directed locally to a specific site or tissue in or on the body, such as the lung, bladder, or prostate. Typically, radiation therapy is administered in pulses over a period of time from about 1 to 2 weeks. The radiation therapy may, however, be administered over longer periods of time. For instance, radiation therapy may be administered to patients having head and neck cancer for about 6 to about 7 weeks. Optionally, the radiation therapy may be administered as a single dose

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or as multiple, sequential doses. The skilled medical practitioner can determine empirically the appropriate dose or doses of radiation therapy useful herein. In accordance with another embodiment of the invention, the antibody or Fc fusion of the present invention and one or more other anti-cancer therapies are employed to treat cancer cells *ex vivo*. It is contemplated that such *ex vivo* treatment may be useful in bone marrow transplantation and particularly, autologous bone marrow transplantation. For instance, treatment of cells or tissue(s) containing cancer cells with antibody or Fc fusion and one or more other anti-cancer therapies, such as described above, can be employed to deplete or substantially deplete the cancer cells prior to transplantation in a recipient patient. It is of course contemplated that the antibodies and Fc fusions of the invention can be employed in combination with still other therapeutic techniques such as surgery.

[111] In an alternate embodiment, the antibodies and Fc fusions of the present invention are administered with a cytokine. By "cytokine" as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-beta; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture, and biologically active equivalents of the native sequence cytokines.

[112] A variety of other therapeutic agents may find use for administration with the antibodies and Fc fusions of the present invention. In one embodiment, the antibody or Fc fusion is administered with an anti-angiogenic agent. By "anti-angiogenic agent" as used herein is meant a compound that blocks, or interferes to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or a protein, for example an antibody, Fc fusion, or cytokine, that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to Vascular Endothelial Growth Factor (VEGF). In an alternate embodiment, the antibody or Fc fusion is administered with a therapeutic

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agent that induces or enhances adaptive immune response, for example an antibody that targets CTLA-4. In an alternate embodiment, the antibody or Fc fusion is administered with a tyrosine kinase inhibitor. By "tyrosine kinase inhibitor" as used herein is meant a molecule that inhibits to some extent tyrosine kinase activity of a tyrosine kinase. Examples of such inhibitors include but are not limited to quinazolines, such as PD 153035, 4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo(2,3-d) pyrimidines; curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lambert); antisense molecules (e.g. those that bind to ErbB-encoding nucleic acid); quinoxalines (US 5,804,396); tryphostins (US 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering A G); pan-ErbB inhibitors such as C1-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (STI571,Gleevec®; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); C1-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in any of the following patent publications: US 5,804,396; PCT WO 99/09016 (American Cyanimid); PCT WO 98/43960 (American Cyanamid); PCT WO 97/38983 (Warner-Lambert); PCT WO 99/06378 (Warner-Lambert); PCT WO 99/06396 (Warner-Lambert); PCT WO 96/30347 (Pfizer, Inc); PCT WO 96/33978 (AstraZeneca); PCT WO96/3397 (AstraZeneca); PCT WO 96/33980 (AstraZeneca), gefitinib (IRESSA™, ZD1839, AstraZeneca), and OSI-774 (Tarceva™, OSI Pharmaceuticals/Genentech).

[113] In an alternate embodiment, the antibody or Fc fusion of the present invention is conjugated or operably linked to another therapeutic compound. The therapeutic compound may be a cytotoxic agent, a chemotherapeutic agent, a toxin, a radioisotope, a cytokine, or other therapeutically active agent. Conjugates of the antibody or Fc fusion and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., 1971, Science 238:1098. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See PCT WO 94/11026. The linker may be a cleavable linker facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al., 1992, Cancer Research 52: 127-131) may be used. Alternatively, the antibody or Fc fusion is operably linked to the therapeutic agent, e.g. by recombinant techniques or peptide synthesis.

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[114] Chemotherapeutic agents that may be useful for conjugation to the antibodies and Fc fusions of the present invention have been described above. In an alternate embodiment, the antibody or Fc fusion is conjugated or operably linked to a toxin, including but not limited to small molecule toxins and enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof. Small molecule toxins include but are not limited to calicheamicin, maytansine (US 5,208,020), trichothene, and CC1065. In one embodiment of the invention, the antibody or Fc fusion is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody or Fc fusion (Chari *et al.*, 1992, *Cancer Research* 52: 127-131) to generate a maytansinoid-antibody or maytansinoid-Fc fusion conjugate. Another conjugate of interest comprises an antibody or Fc fusion conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin that may be used include but are not limited to γ_1^1, $\square_2^1$, $\square_3$, N-acetyl-γ_1^1, PSAG, and Θ^1_1, (Hinman *et al.*, 1993, *Cancer Research* 53:3336-3342; Lode *et al.*, 1998, *Cancer Research* 58:2925-2928) (US 5,714,586; US 5,712,374; US 5,264,586; US 5,773,001). Dolastatin 10 analogs such as auristatin E (AE) and monomethylauristatin E (MMAE) may find use as conjugates for the Fc variants of the present invention (Doronina *et al.*, 2003, *Nat Biotechnol* 21(7):778-84; Francisco *et al.*, 2003 *Blood* 102(4):1458-65). Useful enyzmatically active toxins include but are not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, PCT WO 93/21232. The present invention further contemplates a conjugate or fusion formed between an antibody or Fc fusion of the present invention and a compound with nucleolytic activity, for example a ribonuclease or DNA endonuclease such as a deoxyribonuclease (DNase).

[115] In an alternate embodiment, an antibody or Fc fusion of the present invention may be conjugated or operably linked to a radioisotope to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugate antibodies and Fc fusions. Examples include, but are not limited to, At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, and radioactive isotopes of Lu.

[116] In yet another embodiment, an antibody or Fc fusion of the present invention may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor or Fc fusion-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide). In an alternate

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embodiment, the antibody or Fc fusion is conjugated or operably linked to an enzyme in order to employ Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT). ADEPT may be used by conjugating or operably linking the antibody or Fc fusion to a prodrug-activating enzyme that converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see PCT WO 81/01145) to an active anti-cancer drug. See, for example, PCT WO 88/07378 and US 4,975,278. The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form. Enzymes that are useful in the method of this invention include but are not limited to alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as .beta.-galactosidase and neuramimidase useful for converting glycosylated prodrugs into free drugs; beta-lactamase useful for converting drugs derivatized.with .alpha.-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, for example, Massey, 1987, *Nature* 328: 457-458). Antibody-abzyme and Fc fusion-abzyme conjugates can be prepared for delivery of the abzyme to a tumor cell population. Other modifications of the antibodies and Fc fusions of the present invention are contemplated herein. For example, the antibody or Fc fusion may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

[117] Pharmaceutical compositions are contemplated wherein an antibody or Fc fusion of the present invention and and one or more therapeutically active agents are formulated. Formulations of the antibodies and Fc fusions of the present invention are prepared for storage by mixing said antibody or Fc fusion having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed.,1980), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, acetate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl orbenzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine;

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monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; sweeteners and other flavoring agents; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; additives; coloring agents; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). In a preferred embodiment, the pharmaceutical composition that comprises the antibody or Fc fusion of the present invention is in a water-soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine. The formulations to be used for *in vivo* administration are preferrably sterile. This is readily accomplished by filtration through sterile filtration membranes or other methods.

[118] The antibodies and Fc fusions disclosed herein may also be formulated as immunoliposomes. A liposome is a small vesicle comprising various types of lipids, phospholipids and/or surfactant that is useful for delivery of a therapeutic agent to a mammal. Liposomes containing the antibody or Fc fusion are prepared by methods known in the art, such as described in Epstein *et al.*, 1985, *Proc Natl Acad Sci USA*, 82:3688; Hwang *et al.*, 1980, *Proc Natl Acad Sci USA*, 77:4030; US 4,485,045; US 4,544,545; and PCT WO 97/38731. Liposomes with enhanced circulation time are disclosed in US 5,013,556. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. A chemotherapeutic agent or other therapeutically active agent is optionally contained within the liposome (Gabizon *et al.*, 1989, *J National Cancer Inst* 81:1484).

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[119] The antibodies, Fc fusions, and other therapeutically active agents may also be entrapped in microcapsules prepared by methods including but not limited to coacervation techniques, interfacial polymerization (for example using hydroxymethylcellulose or gelatin-microcapsules, or poly-(methylmethacylate) microcapsules), colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), and macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed., 1980. Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymer, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (US 3,773,919), copolymers of L-glutamic acid and gamma ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (which are injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), poly-D-(-)-3-hydroxybutyric acid, and ProLease® (commercially available from Alkermes), which is a microsphere-based delivery system composed of the desired bioactive molecule incorporated into a matrix of poly-DL-lactide-co-glycolide (PLG).

[120] The concentration of the therapeutically active antibody or Fc fusion of the present invention in the formulation may vary from about 0.1 to 100 weight %. In a preferred embodiment, the concentration of the antibody or Fc fusion is in the range of 0.003 to 1.0 molar. In order to treat a patient, a therapeutically effective dose of the antibody or Fc fusion of the present invention may be administered. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. Dosages may range from 0.01 to 100 mg/kg of body weight or greater, for example 0.1, 1, 10, or 50 mg/kg of body weight, with 1 to 10mg/kg being preferred. As is known in the art, adjustments for antibody or Fc fusion degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

[121] Administration of the pharmaceutical composition comprising an antibody or Fc fusion of the present invention, preferably in the form of a sterile aqueous solution, may be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, intraotically, transdermally, topically (e.g., gels, salves, lotions, creams, etc.), intraperitoneally, intramuscularly, intrapulmonary (e.g., AERx® inhalable technology commercially available from Aradigm, or Inhance™ pulmonary delivery system commercially available from Inhale Therapeutics), vaginally, parenterally, rectally, or intraocularly. In some instances, for example for the treatment of wounds, inflammation, etc., the antibody or Fc fusion may be directly applied as a solution or spray. As is known in the art,

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the pharmaceutical composition may be formulated accordingly depending upon the manner of introduction.

Engineering Methods

[122] The present invention provides engineering methods that may be used to generate Fc variants. A principal obstacle that has hindered previous attempts at Fc engineering is that only random attempts at modification have been possible, due in part to the inefficiency of engineering strategies and methods, and to the low-throughput nature of antibody production and screening. The present invention describes engineering methods that overcome these shortcomings. A variety of design strategies, computational screening methods, library generation methods, and experimental production and screening methods are contemplated. These strategies, approaches, techniques, and methods may be applied individually or in various combinations to engineer optimized Fc variants.

Design Strategies

[123] The most efficient approach to generating Fc variants that are optimized for a desired property is to direct the engineering efforts toward that goal. Accordingly, the present invention teaches design strategies that may be used to engineer optimized Fc variants. The use of a design strategy is meant to guide Fc engineering, but is not meant to constrain an Fc variant to a particular optimized property based on the design strategy that was used to engineer it. At first thought this may seem counterintuitive; however its validity is derived from the enormous complexity of subtle interactions that determine the structure, stability, solubility, and function of proteins and protein-protein complexes. Although efforts can be made to predict which protein positions, residues, interactions, etc. are important for a design goal, often times critical ones are not predictable. Effects on protein structure, stability, solubility, and function, whether favorable or unfavorable, are often unforeseen. Yet there are innumerable amino acid modifications that are detrimental or deleterious to proteins. Thus often times the best approach to engineering comes from generation of protein variants that are focused generally towards a design goal but do not cause detrimental effects. In this way, a principal objective of a design strategy may be the generation of quality diversity. At a simplistic level this can be thought of as stacking the odds in one's favor. As an example, perturbation of the Fc carbohydrate or a particular domain-domain angle, as described below, are valid design strategies for generating optimized Fc variants, despite the fact that how carbohydrate and domain-domain angles determine the properties of Fc is not well understood. By reducing the number of detrimental amino acid modifications that are screened, i.e. by screening quality diversity, these design strategies become practical. Thus the true value of the design strategies taught in the present invention is their ability to direct engineering efforts towards the generation of valuable Fc variants. The specific value of any one resulting variant is determined after experimentation.

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[124] One design strategy for engineering Fc variants is provided in which interaction of Fc with some Fc ligand is altered by engineering amino acid modifications at the interface between Fc and said Fc ligand. Fc ligands herein may include but are not limited to FcγRs, C1q, FcRn, protein A or G, and the like. By exploring energetically favorable substitutions at Fc positions that impact the binding interface, variants can be engineered that sample new interface conformations, some of which may improve binding to the Fc ligand, some of which may reduce Fc ligand binding, and some of which may have other favorable properties. Such new interface conformations could be the result of, for example, direct interaction with Fc ligand residues that form the interface, or indirect effects caused by the amino acid modifications such as perturbation of side chain or backbone conformations. Variable positions may be chosen as any positions that are believed to play an important role in determining the conformation of the interface. For example, variable positions may be chosen as the set of residues that are within a certain distance, for example 5 Angstroms (Å), preferrably between 1 and 10 Å, of any residue that makes direct contact with the Fc ligand.

[125] An additional design strategy for generating Fc variants is provided in which the conformation of the Fc carbohydrate at N297 is optimized. Optimization as used in this context is meant to includes conformational and compositional changes in the N297 carbohydrate that result in a desired property, for example increased or reduced affinity for an FcγR. Such a strategy is supported by the observation that the carbohydrate structure and conformation dramatically affect Fc/FcγR and Fc/C1q binding (Umaña *et al.*, 1999, *Nat Biotechnol* 17:176-180; Davies *et al.*, 2001, *Biotechnol Bioeng* 74:288-294; Mimura *et al.*, 2001, *J Biol Chem* 276:45539-45547.; Radaev *et al.*, 2001, *J Biol Chem* 276:16478-16483; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740; Shinkawa *et al.*, 2003, *J Biol Chem* 278:3466-3473). However the carbohydrate makes no specific contacts with FcγRs. By exploring energetically favorable substitutions at positions that interact with carbohydrate, a quality diversity of variants can be engineered that sample new carbohydrate conformations, some of which may improve and some of which may reduce binding to one or more Fc ligands. While the majority of mutations near the Fc/carbohydrate interface appear to alter carbohydrate conformation, some mutations have been shown to alter the glycosylation composition (Lund *et al.*, 1996, *J Immunol* 157:4963-4969; Jefferis *et al.*, 2002, *Immunol Lett* 82:57-65).

[126] Another design strategy for generating Fc variants is provided in which the angle between the Cγ2 and Cγ3 domains is optimized Optimization as used in this context is meant to describe conformational changes in the Cγ2-Cγ3 domain angle that result in a desired property, for example increased or reduced affinity for an FcγR. This angle is an important determinant of Fc/FcγR affinity (Radaev *et al.*, 2001, *J Biol Chem* 276:16478-16483), and a number of mutations distal to the Fc/FcγR interface affect binding potentially by modulating it (Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604). By exploring energetically favorable substitutions positions that appear to play a key role in determining the Cγ2-Cγ3 angle and the flexibility of the domains relative to one another, a

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quality diversity of variants can be designed that sample new angles and levels of flexibility, some of which may be optimized for a desired Fc property.

[127] Another design strategy for generating Fc variants is provided in which Fc is reengineered to eliminate the structural and functional dependence on glycosylation. This design strategy involves the optimization of Fc structure, stability, solubility, and/or Fc function (for example affinity of Fc for one or more Fc ligands) in the absence of the N297 carbohydrate. In one approach, positions that are exposed to solvent in the absence of glycosylation are engineered such that they are stable, structurally consistent with Fc structure, and have no tendency to aggregate. The Cγ2 is the only unpaired Ig domain in the antibody (see Figure 1). Thus the N297 carbohydrate covers up the exposed hydrophobic patch that would normally be the interface for a protein-protein interaction with another Ig domain, maintaining the stability and structural integrity of Fc and keeping the Cγ2 domains from aggregating across the central axis. Approaches for optimizing aglycosylated Fc may involve but are not limited to designing amino acid modifications that enhance aglycoslated Fc stability and/or solubility by incorporating polar and/or charged residues that face inward towards the Cγ2-Cγ2 dimer axis, and by designing amino acid modifications that directly enhance the aglycosylated Fc/FcγR interface or the interface of aglycosylated Fc with some other Fc ligand.

[128] An additional design strategy for engineering Fc variants is provided in which the conformation of the Cγ2 domain is optimized Optimization as used in this context is meant to describe conformational changes in the Cγ2 domain angle that result in a desired property, for example increased or reduced affinity for an FcγR. By exploring energetically favorable substitutions at Cγ2 positions that impact the Cγ2 conformation, a quality diversity of variants can be engineered that sample new Cγ2 conformations, some of which may achieve the design goal. Such new Cγ2 conformations could be the result of, for example, alternate backbone conformations that are sampled by the variant. Variable positions may be chosen as any positions that are believed to play an important role in determining C⊓2 structure, stability, solubility, flexibility, function, and the like. For example, Cγ2 hydrophobic core residues, that is Cγ2 residues that are partially or fully sequestered from solvent, may be reengineered. Alternatively, noncore residues may be considered, or residues that are deemed important for determining backbone structure, stability, or flexibility.

[129] An additional design strategy for Fc optimization is provided in which binding to an FcγR, complement, or some other Fc ligand is altered by modifications that modulate the electrostatic interaction between Fc and said Fc ligand. Such modifications may be thought of as optimization of the global electrostatic character of Fc, and include replacement of neutral amino acids with a charged amino acid, replacement of a charged amino acid with a neutral amino acid, or replacement of a charged amino acid with an amino acid of opposite charge (i.e. charge reversal). Such

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modifications may be used to effect changes in binding affinity between an Fc and one or more Fc ligands, for example FcγRs. In a preferred embodiment, positions at which electrostatic substitutions might affect binding are selected using one of a variety of well known methods for calculation of electrostatic potentials. In the simplest embodiment, Coulomb's law is used to generate electrostatic potentials as a function of the position in the protein. Additional embodiments include the use of Debye-Huckel scaling to account for ionic strength effects, and more sophisticated embodiments such as Poisson-Boltzmann calculations. Such electrostatic calculations may highlight positions and suggest specific amino acid modifications to achieve the design goal. In some cases, these substitutions may be anticipated to variably affect binding to different Fc ligands, for example to enhance binding to activating FcγRs while decreasing binding affinity to inhibitory FcγRs.

Computational Screening

[130] A principal obstacle to obtaining valuable Fc variants is the difficulty in predicting what amino acid modifications, out of the enormous number of possibilities, will achieve the desired goals. Indeed one of the principle reasons that previous attempts at Fc engineering have failed to produce Fc variants of significant clinical value is that approaches to Fc engineering have thus far involved hit-or-miss approaches. The present invention provides computational screening methods that enable quantitative and systematic engineering of Fc variants. These methods typically use atomic level scoring functions, side chain rotamer sampling, and advanced optimization methods to accurately capture the relationships between protein sequence, structure, and function. Computational screening enables exploration of the entire sequence space of possibilities at target positions by filtering the enormous diversity which results. Variant libraries that are screened computationally are effectively enriched for stable, properly folded, and functional sequences, allowing active optimization of Fc for a desired goal. Because of the overlapping sequence constraints on protein structure, stability, solubility, and function, a large number of the candidates in a library occupy "wasted" sequence space. For example, a large fraction of sequence space encodes unfolded, misfolded, incompletely folded, partially folded, or aggregated proteins. This is particularly relevant for Fc engineering because Ig domains are small beta sheet structures, the engineering of which has proven extremely demanding (Quinn *et al.*, 1994, *Proc Natl Acad Sci U S A* 91:8747-8751; Richardson *et al.*, 2002, *Proc Natl Acad Sci U S A* 99:2754-2759). Even seemingly harmless substitutions on the surface of a beta sheet can cause severe packing conflicts, dramatically disrupting folding equilibrium (Smith *et al.*, 1995, *Science* 270:980-982); incidentally, alanine is one of the worst beta sheet formers (Minor *et al.*, 1994, *Nature* 371:264-267). The determinants of beta sheet stability and specificity are a delicate balance between an extremely large number of subtle interactions. Computational screening enables the generation of libraries that are composed primarily of productive sequence space, and as a result increases the chances of identifying proteins that are optimized for the design goal. In effect, computational screening yields an increased hit-rate, thereby decreasing the number of variants that must be screened experimentally. An additional obstacle to Fc engineering is the need for active design of correlated or coupled mutations. For example, the greatest Fc/FcγR affinity

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enhancement observed thus far is S298A/E333A/K334A, obtained by combining three better binders obtained separately in an alanine scan (Shields *et al.*, 2001, *J Biol Chem* 276:6591-6604). Computational screening is capable of generating such a three-fold variant in one experiment instead of three separate ones, and furthermore is able to test the functionality of all 20 amino acids at those positions instead of just alanine. Computational screening deals with such complexity by reducing the combinatorial problem to an experimentally tractable size.

[131] Computational screening, viewed broadly, has four steps: 1) selection and preparation of the protein template structure or structures, 2) selection of variable positions, amino acids to be considered at those positions, and/or selection of rotamers to model considered amino acids, 3) energy calculation, and 4) combinatorial optimization. In more detail, the process of computational screening can be described as follows. A three-dimensional structure of a protein is used as the starting point. The positions to be optimized are identified, which may be the entire protein sequence or subset(s) thereof. Amino acids that will be considered at each position are selected. In a preferred embodiment, each considered amino acid may be represented by a discrete set of allowed conformations, called rotamers. Interaction energies are calculated between each considered amino acid and each other considered amino acid, and the rest of the protein, including the protein backbone and invariable residues. In a preferred embodiment, interaction energies are calculated between each considered amino acid side chain rotamer and each other considered amino acid side chain rotamer and the rest of the protein, including the protein backbone and invariable residues. One or more combinatorial search algorithms are then used to identify the lowest energy sequence and/or low energy sequences.

[132] In a preferred embodiment, the computational screening method used is substantially similar to Protein Design Automation® (PDA®) technology, as is described in US 6,188,965; US 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588. In another preferred embodiment, a computational screening method substantially similar to Sequence Prediction Algorithm™ (SPA™) technology is used, as is described in (Raha *et al.*, 2000, *Protein Sci* 9:1106-1119), USSN 09/877,695, and USSN 10/071,859. In another preferred embodiment, the computational screening methods described in USSN 10/339788, filed on March 3, 2003, entitled "ANTIBODY OPTIMIZATION", are used. In some embodiments, combinations of different computational screening methods are used, including combinations of PDA® technology and SPA™ technology, as well as combinations of these computational methods in combination with other design tools. Similarly, these computational methods can be used simultaneously or sequentially, in any order.

[133] A template structure is used as input into the computational screening calculations. By "template structure" herein is meant the structural coordinates of part or all of a protein to be

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optimized. The template structure may be any protein for which a three dimensional structure (that is, three dimensional coordinates for a set of the protein's atoms) is known or may be calculated, estimated, modeled, generated, or determined. The three dimensional structures of proteins may be determined using methods including but not limited to X-ray crystallographic techniques, nuclear magnetic resonance (NMR) techniques, *de novo* modeling, and homology modeling. If optimization is desired for a protein for which the structure has not been solved experimentally, a suitable structural model may be generated that may serve as the template for computational screening calculations. Methods for generating homology models of proteins are known in the art, and these methods find use in the present invention. See for example, Luo, *et al.* 2002, *Protein Sci* 11: 1218-1226, Lehmann & Wyss, 2001, *Curr Opin Biotechnol* 12(4):371-5.; Lehmann *et al.*, 2000, Biochim Biophys Acta 1543(2):408-415; Rath & Davidson, 2000, *Protein Sci*, 9(12):2457-69; Lehmann *et al.*, 2000, *Protein Eng* 13(1):49-57; Desjarlais & Berg, 1993, *Proc Natl Acad Sci USA* 90(6):2256-60; Desjarlais & Berg, 1992, *Proteins* 12(2):101-4; Henikoff & Henikoff, 2000, *Adv Protein Chem* 54:73-97; Henikoff & Henikoff, 1994, *J Mol Biol* 243(4):574-8; Morea *et al.*, 2000, *Methods* 20:267-269. Protein/protein complexes may also be obtained using docking methods. Suitable protein structures that may serve as template structures include, but are not limited to, all of those found in the Protein Data Base compiled and serviced by the Research Collaboratory for Structural Bioinformatics (RCSB, formerly the Brookhaven National Lab).

[134] The template structure may be of a protein that occurs naturally or is engineered. The template structure may be of a protein that is substantially encoded by a protein from any organism, with human, mouse, rat, rabbit, and monkey preferred. The template structure may comprise any of a number of protein structural forms. In a preferred embodiment the template structure comprises an Fc region or a domain or fragment of Fc. In an alternately preferred embodiment the template structure comprises Fc or a domain or fragment of Fc bound to one or more Fc ligands, with an Fc/FcγR complex being preferred. The Fc in the template structure may be glycosylated or unglycosylated. The template structure may comprise more than one protein chain. The template structure may additionally contain nonprotein components, including but not limited to small molecules, substrates, cofactors, metals, water molecules, prosthetic groups, polymers and carbohydrates. In a preferred embodiment, the template structure is a plurality or set of template proteins, for example an ensemble of structures such as those obtained from NMR. Alternatively, the set of template structures is generated from a set of related proteins or structures, or artificially created ensembles. The composition and source of the template structure depends on the engineering goal. For example, for enhancement of human Fc/FcγR affinity, a human Fc/FcγR complex structure or derivative thereof may be used as the template structure. Alternatively, the uncomplexed Fc structure may be used as the template structure. If the goal is to enhance affinity of a human Fc for a mouse FcγR, the template structure may be a structure or model of a human Fc bound to a mouse FcγR.

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[135] The template structure may be modified or altered prior to design calculations. A variety of methods for template structure preparation are described in US 6,188,965; US 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 09/877,695; USSN 10/071,859, USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588. For example, in a preferred embodiment, explicit hydrogens may be added if not included within the structure. In an alternate embodiment, energy minimization of the structure is run to relax strain, including strain due to van der Waals clashes, unfavorable bond angles, and unfavorable bond lengths. Alternatively, the template structure is altered using other methods, such as manually, including directed or random perturbations. It is also possible to modify the template structure during later steps of computational screening, including during the energy calculation and combinatorial optimization steps. In an alternate embodiment, the template structure is not modified before or during computational screening calculations.

[136] Once a template structure has been obtained, variable positions are chosen. By "variable position" herein is meant a position at which the amino acid identity is allowed to be altered in a computational screening calculation. As is known in the art, allowing amino acid modifications to be considered only at certain variable positions reduces the complexity of a calculation and enables computational screening to be more directly tailored for the design goal. One or more residues may be variable positions in computational screening calculations. Positions that are chosen as variable positions may be those that contribute to or are hypothesized to contribute to the protein property to be optimized, for example Fc affinity for an FcγR, Fc stability, Fc solubility, and so forth. Residues at variable positions may contribute favorably or unfavorably to a specific protein property. For example, a residue at an Fc/FcγR interface may be involved in mediating binding, and thus this position may be varied in design calculations aimed at improving Fc/FcγR affinity. As another example, a residue that has an exposed hydrophobic side chain may be responsible for causing unfavorable aggregation, and thus this position may be varied in design calculations aimed at improving solubility. Variable positions may be those positions that are directly involved in interactions that are determinants of a particular protein property. For example, the Fc□R binding site of Fc may be defined to include all residues that contact that particular FγcR. By "contact" herein is meant some chemical interaction between at least one atom of an Fc residue with at least one atom of the bound FcγR, with chemical interaction including, but not limited to van der Waals interactions, hydrogen bond interactions, electrostatic interactions, and hydrophobic interactions. In an alternative embodiment, variable positions may include those positions that are indirectly involved in a protein property, i.e. such positions may be proximal to residues that are known to or hypothesized to contribute to an Fc property. For example, the FcγR binding site of an Fc may be defined to include all Fc residues within a certain distance, for example 4 - 10 Å, of any Fc residue that is in van der Waals contact with the FcγR. Thus variable positions in this case may be chosen not only as residues that directly contact the FcγR, but also those that contact residues that contact the FcγR and thus influence binding indirectly. The specific positions chosen are dependent on the design strategy being employed.

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[137] One or more positions in the template structure that are not variable may be floated. By "floated position" herein is meant a position at which the amino acid conformation but not the amino acid identity is allowed to vary in a computational screening calculation. In one embodiment, the floated position may have the parent amino acid identity. For example, floated positions may be positions that are within a small distance, for example 5 Å, of a variable position residue. In an alternate embodiment, a floated position may have a non-parent amino acid identity. Such an embodiment may find use in the present invention, for example, when the goal is to evaluate the energetic or structural outcome of a specific mutation.

[138] Positions that are not variable or floated are fixed. By "fixed position" herein is meant a position at which the amino acid identity and the conformation are held constant in a computational screening calculation. Positions that may be fixed include residues that are not known to be or hypothesized to be involved in the property to be optimized. In this case the assumption is that there is little or nothing to be gained by varying these positions. Positions that are fixed may also include positions whose residues are known or hypothesized to be important for maintaining proper folding, structure, stability, solubility, and/or biological function. For example, positions may be fixed for residues that interact with a particular Fc ligand or residues that encode a glycosylation site in order to ensure that binding to the Fc ligand and proper glycosylation respectively are not perturbed. Likewise, if stability is being optimized, it may be beneficial to fix positions that directly or indirectly interact with an Fc ligand, for example an Fc□R, so that binding is not perturbed. Fixed positions may also include structurally important residues such as cysteines participating in disulfide bridges, residues critical for determining backbone conformation such as proline or glycine, critical hydrogen bonding residues, and residues that form favorable packing interactions.

[139] The next step in computational screening is to select a set of possible amino acid identities that will be considered at each particular variable position. This set of possible amino acids is herein referred to as "considered amino acids" at a variable position. "Amino acids" as used herein refers to the set of natural 20 amino acids and any nonnatural or synthetic analogues. In one embodiment, all 20 natural amino acids are considered. Alternatively, a subset of amino acids, or even only one amino acid is considered at a given variable position. As will be appreciated by those skilled in the art, there is a computational benefit to considering only certain amino acid identities at variable positions, as it decreases the combinatorial complexity of the search. Furthermore, considering only certain amino acids at variable positions may be used to tailor calculations toward specific design strategies. For example, for solubility optimization of aglycosylated Fc, it may be beneficial to allow only polar amino acids to be considered at nonpolar Fc residues that are exposed to solvent in the absence of carbohydrate. Nonnatural amino acids, including synthetic amino acids and analogues of natural amino acids, may also be considered amino acids. For example see Chin *et al.*, 2003, *Science*, 301(5635):964-7; and Chin *et al.*, 2003, *Chem Biol.*10(6):511-9.

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[140] A wide variety of methods may be used, alone or in combination, to select which amino acids will be considered at each position. For example, the set of considered amino acids at a given variable position may be chosen based on the degree of exposure to solvent. Hydrophobic or nonpolar amino acids typically reside in the interior or core of a protein, which are inaccessible or nearly inaccessible to solvent. Thus at variable core positions it may be beneficial to consider only or mostly nonpolar amino acids such as alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine. Hydrophilic or polar amino acids typically reside on the exterior or surface of proteins, which have a significant degree of solvent accessibility. Thus at variable surface positions it may be beneficial to consider only or mostly polar amino acids such as alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine and histidine. Some positions are partly exposed and partly buried, and are not clearly protein core or surface positions, in a sense serving as boundary residues between core and surface residues. Thus at such variable boundary positions it may be beneficial to consider both nonpolar and polar amino acids such as alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine histidine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine. Determination of the degree of solvent exposure at variable positions may be by subjective evaluation or visual inspection of the template structure by one skilled in the art of protein structural biology, or by using a variety of algorithms that are known in the art. Selection of amino acid types to be considered at variable positions may be aided or determined wholly by computational methods, such as calculation of solvent accessible surface area, or using algorithms that assess the orientation of the C□-C□ vectors relative to a solvent accessible surface, as outlined in US 6,188,965; 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588. In one embodiment, each variable position may be classified explicitly as a core, surface, or boundary position or a classification substantially similar to core, surface, or boundary.

[141] In an alternate embodiment, selection of the set of amino acids allowed at variable positions may be hypothesis-driven. Hypotheses for which amino acid types should be considered at variable positions may be derived by a subjective evaluation or visual inspection of the template structure by one skilled in the art of protein structural biology. For example, if it is suspected that a hydrogen bonding interaction may be favorable at a variable position, polar residues that have the capacity to form hydrogen bonds may be considered, even if the position is in the core. Likewise, if it is suspected that a hydrophobic packing interaction may be favorable at a variable position, nonpolar residues that have the capacity to form favorable packing interactions may be considered, even if the position is on the surface. Other examples of hypothesis-driven approaches may involve issues of backbone flexibility or protein fold. As is known in the art, certain residues, for example proline, glycine, and cysteine, play important roles in protein structure and stability. Glycine enables greater backbone flexibility than all other amino acids, proline constrains the backbone more than all other

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amino acids, and cysteines may form disulfide bonds. It may therefore be beneficial to include one or more of these amino acid types to achieve a desired design goal. Alternatively, it may be beneficial to exclude one or more of these amino acid types from the list of considered amino acids.

[142] In an alternate embodiment, subsets of amino acids may be chosen to maximize coverage. In this case, additional amino acids with properties similar to that in the template structure may be considered at variable positions. For example, if the residue at a variable position in the template structure is a large hydrophobic residue, additional large hydrophobic amino acids may be considered at that position. Alternatively, subsets of amino acids may be chosen to maximize diversity. In this case, amino acids with properties dissimilar to those in the template structure may be considered at variable positions. For example, if the residue at a variable position in the template is a large hydrophobic residue, amino acids that are small, polar, etc. may be considered.

[143] As is known in the art, some computational screening methods require only the identity of considered amino acids to be determined during design calculations. That is, no information is required concerning the conformations or possible conformations of the amino acid side chains. Other preferred methods utilize a set of discrete side chain conformations, called rotamers, which are considered for each amino acid. Thus, a set of rotamers may be considered at each variable and floated position. Rotamers may be obtained from published rotamer libraries (see for example, Lovel *et al.*, 2000, *Proteins: Structure Function and Genetics* 40:389-408; Dunbrack & Cohen, 1997, *Protein Science* 6:1661-1681; DeMaeyer *et al.*, 1997, *Folding and Design* 2:53-66; Tuffery *et al.*, 1991, *J Biomol Struct Dyn* 8:1267-1289, Ponder & Richards, 1987, *J Mol Biol* 193:775-791). As is known in the art, rotamer libraries may be backbone-independent or backbone-dependent. Rotamers may also be obtained from molecular mechanics or *ab initio* calculations, and using other methods. In a preferred embodiment, a flexible rotamer model is used (see Mendes *et al.*, 1999, *Proteins: Structure, Function, and Genetics* 37:530-543). Similarly, artificially generated rotamers may be used, or augment the set chosen for each amino acid and/or variable position. In one embodiment, at least one conformation that is not low in energy is included in the list of rotamers. In an alternate embodiment, the rotamer of the variable position residue in the template structure is included in the list of rotamers allowed for that variable position. In an alternate embodiment, only the identity of each amino acid considered at variable positions is provided, and no specific conformational states of each amino acid are used during design calculations. That is, use of rotamers is not essential for computational screening.

[144] Experimental information may be used to guide the choice of variable positions and/or the choice of considered amino acids at variable positions. As is known in the art, mutagenesis experiments are often carried out to determine the role of certain residues in protein structure and function, for example, which protein residues play a role in determining stability, or which residues

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make up the interface of a protein-protein interaction. Data obtained from such experiments are useful in the present invention. For example, variable positions for Fc/FcγR affinity enhancement could involve varying all positions at which mutation has been shown to affect binding. Similarly, the results from such an experiment may be used to guide the choice of allowed amino acid types at variable positions. For example, if certain types of amino acid substitutions are found to be favorable, similar types of those amino acids may be considered. In one embodiment, additional amino acids with properties similar to those that were found to be favorable experimentally may be considered at variable positions. For example, if experimental mutation of a variable position at an Fc/FcγR interface to a large hydrophobic residue was found to be favorable, the user may choose to include additional large hydrophobic amino acids at that position in the computational screen. As is known in the art, display and other selection technologies may be coupled with random mutagenesis to generate a list or lists of amino acid substitutions that are favorable for the selected property. Such a list or lists obtained from such experimental work find use in the present invention. For example, positions that are found to be invariable in such an experiment may be excluded as variable positions in computational screening calculations, whereas positions that are found to be more acceptable to mutation or respond favorably to mutation may be chosen as variable positions. Similarly, the results from such experiments may be used to guide the choice of allowed amino acid types at variable positions. For example, if certain types of amino acids arise more frequently in an experimental selection, similar types of those amino acids may be considered. In one embodiment, additional amino acids with properties similar to those that were found to be favorable experimentally may be considered at variable positions. For example, if selected mutations at a variable position that resides at an Fc/FcγR interface are found to be uncharged polar amino acids, the user may choose to include additional uncharged polar amino acids, or perhaps charged polar amino acids, at that position.

[145] Sequence information may also be used to guide choice of variable positions and/or the choice of amino acids considered at variable positions. As is known in the art, some proteins share a common structural scaffold and are homologous in sequence. This information may be used to gain insight into particular positions in the protein family. As is known in the art, sequence alignments are often carried out to determine which protein residues are conserved and which are not conserved. That is to say, by comparing and contrasting alignments of protein sequences, the degree of variability at a position may be observed, and the types of amino acids that occur naturally at positions may be observed. Data obtained from such analyses are useful in the present invention. The benefit of using sequence information to choose variable positions and considered amino acids at variable positions are several fold. For choice of variable positions, the primary advantage of using sequence information is that insight may be gained into which positions are more tolerant and which are less tolerant to mutation. Thus sequence information may aid in ensuring that quality diversity, i.e. mutations that are not deleterious to protein structure, stability, etc., is sampled computationally. The same advantage applies to use of sequence information to select amino acid types considered at variable positions. That is, the set of amino acids that occur in a protein sequence alignment may be

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thought of as being pre-screened by evolution to have a higher chance than random for being compatible with a protein's structure, stability, solubility, function, etc. Thus higher quality diversity is sampled computationally. A second benefit of using sequence information to select amino acid types considered at variable positions is that certain alignments may represent sequences that may be less immunogenic than random sequences. For example, if the amino acids considered at a given variable position are the set of amino acids which occur at that position in an alignment of human protein sequences, those amino acids may be thought of as being pre-screened by nature for generating no or low immune response if the optimized protein is used as a human therapeutic.

[146] The source of the sequences may vary widely, and include one or more of the known databases, including but not limited to the Kabat database (Johnson & Wu, 2001, *Nucleic Acids Res* 29:205-206; Johnson & Wu, 2000, *Nucleic Acids Res* 28:214-218), the IMGT database (IMGT, the international ImMunoGeneTics information system®; Lefranc *et al.*, 1999, *Nucleic Acids Res* 27:209-212; Ruiz *et al.*, 2000 *Nucleic Acids Re.* 28:219-221; Lefranc *et al.*, 2001, *Nucleic Acids Res* 29:207-209; Lefranc *et al.*, 2003, *Nucleic Acids Res* 31:307-310), and VBASE, SwissProt, GenBank and Entrez, and EMBL Nucleotide Sequence Database. Protein sequence information can be obtained, compiled, and/or generated from sequence alignments of naturally occurring proteins from any organism, including but not limited to mammals. Protein sequence information can be obtained from a database that is compiled privately. There are numerous sequence-based alignment programs and methods known in the art, and all of these find use in the present invention for generation of sequence alignments of proteins that comprise Fc and Fc ligands.

[147] Once alignments are made, sequence information can be used to guide choice of variable positions. Such sequence information can relate the variability, natural or otherwise, of a given position. Variability herein should be distinguished from variable position. Variability refers to the degree to which a given position in a sequence alignment shows variation in the types of amino acids that occur there. Variable position, to reiterate, is a position chosen by the user to vary in amino acid identity during a computational screening calculation. Variability may be determined qualitatively by one skilled in the art of bioinformatics. There are also methods known in the art to quantitatively determine variability that may find use in the present invention. The most preferred embodiment measures Information Entropy or Shannon Entropy. Variable positions can be chosen based on sequence information obtained from closely related protein sequences, or sequences that are less closely related.

[148] The use of sequence information to choose variable positions finds broad use in the present invention. For example, if an Fc/FcγR interface position in the template structure is tryptophan, and tryptophan is observed at that position in greater than 90% of the sequences in an alignment, it may be beneficial to leave that position fixed. In contrast, if another interface position is found to have a

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greater level of variability, for example if five different amino acids are observed at that position with frequencies of approximately 20% each, that position may be chosen as a variable position. In another embodiment, visual inspection of aligned protein sequences may substitute for or aid visual inspection of a protein structure. Sequence information can also be used to guide the choice of amino acids considered at variable positions. Such sequence information can relate to how frequently an amino acid, amino acids, or amino acid types (for example polar or nonpolar, charged or uncharged) occur, naturally or otherwise, at a given position. In one embodiment, the set of amino acids considered at a variable position may comprise the set of amino acids that is observed at that position in the alignment. Thus, the position-specific alignment information is used directly to generate the list of considered amino acids at a variable position in a computational screening calculation. Such a strategy is well known in the art; see for example Lehmann & Wyss, 2001, *Curr Opin Biotechnol* 12(4):371-5; Lehmann *et al.*, 2000, *Biochim Biophys Acta* 1543(2):408-415; Rath & Davidson, 2000, *Protein Sci*, 9(12):2457-69; Lehmann *et al.*, 2000, *Protein Eng* 13(1):49-57; Desjarlais & Berg, 1993, *Proc Natl Acad Sci USA* 90(6):2256-60; Desjarlais & Berg, 1992, *Proteins* 12(2):101-4; Henikoff & Henikoff, 2000, *Adv Protein Chem* 54:73-97; Henikoff & Henikoff, 1994, *J Mol Biol* 243(4):574-8. In an alternate embodiment, the set of amino acids considered at a variable position or positions may comprise a set of amino acids that is observed most frequently in the alignment. Thus, a certain criteria is applied to determine whether the frequency of an amino acid or amino acid type warrants its inclusion in the set of amino acids that are considered at a variable position. As is known in the art, sequence alignments may be analyzed using statistical methods to calculate the sequence diversity at any position in the alignment and the occurrence frequency or probability of each amino acid at a position. Such data may then be used to determine which amino acids types to consider. In the simplest embodiment, these occurrence frequencies are calculated by counting the number of times an amino acid is observed at an alignment position, then dividing by the total number of sequences in the alignment. In other embodiments, the contribution of each sequence, position or amino acid to the counting procedure is weighted by a variety of possible mechanisms. In a preferred embodiment, the contribution of each aligned sequence to the frequency statistics is weighted according to its diversity weighting relative to other sequences in the alignment. A common strategy for accomplishing this is the sequence weighting system recommended by Henikoff and Henikoff (Henikoff & Henikoff, 2000, *Adv Protein Chem* 54:73-97; Henikoff & Henikoff, 1994, *J Mol Biol* 243:574-8. In a preferred embodiment, the contribution of each sequence to the statistics is dependent on its extent of similarity to the target sequence, i.e. the template structure used, such that sequences with higher similarity to the target sequence are weighted more highly. Examples of similarity measures include, but are not limited to, sequence identity, BLOSUM similarity score, PAM matrix similarity score, and BLAST score. In an alternate embodiment, the contribution of each sequence to the statistics is dependent on its known physical or functional properties. These properties include, but are not limited to, thermal and chemical stability, contribution to activity, and solubility. For example, when optimizing aglycosylated Fc for solubility, those sequences in an alignment that are known to be most soluble (for example see Ewert *et al.*, 2003, *J Mol Biol* 325:531-553), will contribute more heavily to the calculated frequencies.

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[149] Regardless of what criteria are applied for choosing the set of amino acids in a sequence alignment to be considered at variable positions, use of sequence information to choose considered amino acids finds broad use in the present invention. For example, to optimize Fc solubility by replacing exposed nonpolar surface residues, considered amino acids may be chosen as the set of amino acids, or a subset of those amino acids which meet some criteria, that are observed at that position in an alignment of protein sequences. As another example, one or more amino acids may be added or subtracted subjectively from a list of amino acids derived from a sequence alignment in order to maximize coverage. For example, additional amino acids with properties similar to those that are found in a sequence alignment may be considered at variable positions. For example, if an Fc position that is known to or hypothesized to bind an FcγR is observed to have uncharged polar amino acids in a sequence alignment, the user may choose to include additional uncharged polar amino acids in a computational screening calculation, or perhaps charged polar amino acids, at that position.

[150] In one embodiment, sequence alignment information is combined with energy calculation, as discussed below. For example, pseudo energies can be derived from sequence information to generate a scoring function. The use of a sequence-based scoring function may assist in significantly reducing the complexity of a calculation. However, as is appreciated by those skilled in the art, the use of a sequence-based scoring function alone may be inadequate because sequence information can often indicate misleading correlations between mutations that may in reality be structurally conflicting. Thus, in a preferred embodiment, a structure-based method of energy calculation is used, either alone or in combination with a sequence-based scoring function. That is, preferred embodiments do not rely on sequence alignment information alone as the analysis step.

[151] Energy calculation refers to the process by which amino acid modifications are scored. The energies of interaction are measured by one or more scoring functions. A variety of scoring functions find use in the present invention for calculating energies. Scoring functions may include any number of potentials, herein referred to as the energy terms of a scoring function, including but not limited to a van der Waals potential, a hydrogen bond potential, an atomic solvation potential or other solvation models, a secondary structure propensity potential, an electrostatic potential, a torsional potential, and an entropy potential. At least one energy term is used to score each variable or floated position, although the energy terms may differ depending on the position, considered amino acids, and other considerations. In one embodiment, a scoring function using one energy term is used. In the most preferred embodiment, energies are calculated using a scoring function that contains more than one energy term, for example describing van der Waals, solvation, electrostatic, and hydrogen bond interactions, and combinations thereof. In additional embodiments, additional energy terms include but are not limited to entropic terms, torsional energies, and knowledge-based energies.

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[152] A variety of scoring functions are described in US 6,188,965; US 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 09/877,695; USSN 10/071,859, USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588. As will be appreciated by those skilled in the art, scoring functions need not be limited to physico-chemical energy terms. For example, knowledge-based potentials may find use in the computational screening methodology of the present invention. Such knowledge-based potentials may be derived from protein sequence and/or structure statistics including but not limited to threading potentials, reference energies, pseudo energies, homology-based energies, and sequence biases derived from sequence alignments. In a preferred embodiment, a scoring function is modified to include models for immunogenicity, such as functions derived from data on binding of peptides to MHC (Major Htocompatability Complex), that may be used to identify potentially immunogenic sequences (see for example USSN 09/903,378; USSN 10/039,170; USSN 60/222,697; USSN 10/339788; PCT WO 01/21823; and PCT WO 02/00165). In one embodiment, sequence alignment information can be used to score amino acid substitutions. For example, comparison of protein sequences, regardless of whether the source of said proteins is human, monkey, mouse, or otherwise, may be used to suggest or score amino acid mutations in the computational screening methodology of the present invention. In one embodiment, as is known in the art, one or more scoring functions may be optimized or "trained" during the computational analysis, and then the analysis re-run using the optimized system. Such altered scoring functions may be obtained for example, by training a scoring function using experimental data. As will be appreciated by those skilled in the art, a number of force fields, which are comprised of one or more energy terms, may serve as scoring functions. Force fields include but are not limited to *ab initio* or quantum mechanical force fields, semi-empirical force fields, and molecular mechanics force fields. Scoring functions that are knowledge-based or that use statistical methods may find use in the present invention. These methods may be used to assess the match between a sequence and a three-dimensional protein structure, and hence may be used to score amino acid substitutions for fidelity to the protein structure. In one embodiment, molecular dynamics calculations may be used to computationally screen sequences by individually calculating mutant sequence scores.

[153] There are a variety of ways to represent amino acids in order to enable efficient energy calculation. In a preferred embodiment, considered amino acids are represented as rotamers, as described previously, and the energy (or score) of interaction of each possible rotamer at each variable and floated position with the other variable and floated rotamers, with fixed position residues, and with the backbone structure and any non-protein atoms, is calculated. In a preferred embodiment, two sets of interaction energies are calculated for each side chain rotamer at every variable and floated position: the interaction energy between the rotamer and the fixed atoms (the "singles" energy), and the interaction energy between the variable and floated positions rotamer and all other possible rotamers at every other variable and floated position (the "doubles" energy). In an alternate embodiment, singles and doubles energies are calculated for fixed positions as well as for variable and floated positions. In an alternate embodiment, considered amino acids are not represented as rotamers.

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[154] An important component of computational screening is the identification of one or more sequences that have a favorable score, i.e. are low in energy. Determining a set of low energy sequences from an extremely large number of possibilities is nontrivial, and to solve this problem a combinatorial optimization algorithm is employed. The need for a combinatorial optimization algorithm is illustrated by examining the number of possibilities that are considered in a typical computational screening calculation. The discrete nature of rotamer sets allows a simple calculation of the number of possible rotameric sequences for a given design problem. A backbone of length n with m possible rotamers per position will have m^n possible rotamer sequences, a number that grows exponentially with sequence length. For very simple calculations, it is possible to examine each possible sequence in order to identify the optimal sequence and/or one or more favorable sequences. However, for a typical design problem, the number of possible sequences (up to 10^{80} or more) is sufficiently large that examination of each possible sequence is intractable. A variety of combinatorial optimization algorithms may then be used to identify the optimum sequence and/or one or more favorable sequences. Combinatorial optimization algorithms may be divided into two classes: (1) those that are guaranteed to return the global minimum energy configuration if they converge, and (2) those that are not guaranteed to return the global minimum energy configuration, but which will always return a solution. Examples of the first class of algorithms include but are not limited to Dead-End Elimination (DEE) and Branch & Bound (B&B) (including Branch and Terminate) (Gordon & Mayo, 1999, *Structure Fold Des* 7:1089-98). Examples of the second class of algorithms include, but are not limited to, Monte Carlo (MC), self-consistent mean field (SCMF), Boltzmann sampling (Metropolis *et al.*, 1953, *J Chem Phys* 21:1087), simulated annealing (Kirkpatrick *et al.*, 1983, *Science*, 220:671-680), genetic algorithm (GA), and Fast and Accurate Side-Chain Topology and Energy Refinement (FASTER) (Desmet, et al., 2002, Proteins, 48:31-43). A combinatorial optimization algorithm may be used alone or in conjunction with another combinatorial optimization algorithm.

[155] In one embodiment of the present invention, the strategy for applying a combinatorial optimization algorithm is to find the global minimum energy configuration. In an alternate embodiment, the strategy is to find one or more low energy or favorable sequences. In an alternate embodiment, the strategy is to find the global minimum energy configuration and then find one or more low energy or favorable sequences. For example, as outlined in USSN 6,269,312, preferred embodiments utilize a Dead End Elimination (DEE) step and a Monte Carlo step. In other embodiments, tabu search algorithms are used or combined with DEE and/or Monte Carlo, among other search methods (see Modern Heuristic Search Methods, edited by V.J. Rayward-Smith *et al.*, 1996, John Wiley & Sons Ltd.; USSN 10/218,102; and PCT WO 02/25588). In another preferred embodiment, a genetic algorithm may be used; see for example USSN 09/877,695 and USSN 10/071,859. As another example, as is more fully described in US 6,188,965; US 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588, the global optimum may be reached, and then further

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computational processing may occur, which generates additional optimized sequences. In the simplest embodiment, design calculations are not combinatorial. That is, energy calculations are used to evaluate amino acid substitutions individually at single variable positions. For other calculations it is preferred to evaluate amino acid substitutions at more than one variable position. In a preferred embodiment, all possible interaction energies are calculated prior to combinatorial optimization. In an alternatively preferred embodiment, energies may be calculated as needed during combinatorial optimization.

Library generation

[156] The present invention provides methods for generating libraries that may subsequently be screened experimentally to single out optimized Fc variants. By "library" as used herein is meant a set of one or more Fc variants. Library may refer to the set of variants in any form. In one embodiment, the library is a list of nucleic acid or amino acid sequences, or a list of nucleic acid or amino acid substitutions at variable positions. For example, the examples used to illustrate the present invention below provide libraries as amino acid substitutions at variable positions. In one embodiment, a library is a list of at least one sequence that are Fc variants optimized for a desired property. For example see, Filikov *et al.*, 2002, *Protein Sci* 11:1452-1461 and Luo *et al.*, 2002, *Protein Sci* 11:1218-1226. In an alternate embodiment, a library may be defined as a combinatorial list, meaning that a list of amino acid substitutions is generated for each variable position, with the implication that each substitution is to be combined with all other designed substitutions at all other variable positions. In this case, expansion of the combination of all possibilities at all variable positions results in a large explicitly defined library. A library may refer to a physical composition of polypeptides that comprise the Fc region or some domain or fragment of the Fc region. Thus a library may refer to a physical composition of antibodies or Fc fusions, either in purified or unpurified form. A library may refer to a physical composition of nucleic acids that encode the library sequences. Said nucleic acids may be the genes encoding the library members, the genes encoding the library members with any operably linked nucleic acids, or expression vectors encoding the library members together with any other operably linked regulatory sequences, selectable markers, fusion constructs, and/or other elements. For example, the library may be a set of mammalian expression vectors that encode Fc library members, the protein products of which may be subsequently expressed, purified, and screened experimentally. As another example, the library may be a display library. Such a library could, for example, comprise a set of expression vectors that encode library members operably linked to some fusion partner that enables phage display, ribosome display, yeast display, bacterial surface display, and the like.

[157] The library may be generated using the output sequence or sequences from computational screening. As discussed above, computationally generated libraries are significantly enriched in stable, properly folded, and functional sequences relative to randomly generated libraries. As a result, computational screening increases the chances of identifying proteins that are optimized for the

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design goal. The set of sequences in a library is generally, but not always, significantly different from the parent sequence, although in some cases the library preferably contains the parent sequence. As is known in the art, there are a variety of ways that a library may be derived from the output of computational screening calculations. For example, methods of library generation described in US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 01/40091; and PCT WO 02/25588 find use in the present invention. In one embodiment, sequences scoring within a certain range of the global optimum sequence may be included in the library. For example, all sequences within 10 kcal/mol of the lowest energy sequence could be used as the library. In an alternate embodiment, sequences scoring within a certain range of one or more local minima sequences may be used. In a preferred embodiment, the library sequences are obtained from a filtered set. Such a list or set may be generated by a variety of methods, as is known in the art, for example using an algorithm such as Monte Carlo, B&B, or SCMF. For example, the top 10^3 or the top 10^5 sequences in the filtered set may comprise the library. Alternatively, the total number of sequences defined by the combination of all mutations may be used as a cutoff criterion for the library. Preferred values for the total number of recombined sequences range from 10 to 10^{20}, particularly preferred values range from 100 to 10^9. Alternatively, a cutoff may be enforced when a predetermined number of mutations per position is reached. In some embodiments, sequences that do not make the cutoff are included in the library. This may be desirable in some situations, for instance to evaluate the approach to library generation, to provide controls or comparisons, or to sample additional sequence space. For example, the parent sequence may be included in the library, even if it does not make the cutoff.

[158] Clustering algorithms may be useful for classifying sequences derived by computational screening methods into representative groups. For example, the methods of clustering and their application described in USSN 10/218,102 and PCT WO 02/25588, find use in the present invention. Representative groups may be defined, for example, by similarity. Measures of similarity include, but are not limited to sequence similarity and energetic similarity. Thus the output sequences from computational screening may be clustered around local minima, referred to herein as clustered sets of sequences. For example, sets of sequences that are close in sequence space may be distinguished from other sets. In one embodiment, coverage within one or a subset of clustered sets may be maximized by including in the library some, most, or all of the sequences that make up one or more clustered sets of sequences. For example, it may be advantageous to maximize coverage within the one, two, or three lowest energy clustered sets by including the majority of sequences within these sets in the library. In an alternate embodiment, diversity across clustered sets of sequences may be sampled by including within a library only a subset of sequences within each clustered set. For example, all or most of the clustered sets could be broadly sampled by including the lowest energy sequence from each clustered set in the library.

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[159] Sequence information may be used to guide or filter computationally screening results for generation of a library. As discussed, by comparing and contrasting alignments of protein sequences, the degree of variability at a position and the types of amino acids which occur naturally at that position may be observed. Data obtained from such analyses are useful in the present invention. The benefits of using sequence information have been discussed, and those benefits apply equally to use of sequence information to guide library generation. The set of amino acids that occur in a sequence alignment may be thought of as being pre-screened by evolution to have a higher chance than random at being compatible with a protein's structure, stability, solubility, function, and immunogenicity. The variety of sequence sources, as well as the methods for generating sequence alignments that have been discussed, find use in the application of sequence information to guiding library generation. Likewise, as discussed above, various criteria may be applied to determine the importance or weight of certain residues in an alignment. These methods also find use in the application of sequence information to guide library generation. Using sequence information to guide library generation from the results of computational screening finds broad use in the present invention. In one embodiment, sequence information is used to filter sequences from computational screening output. That is to say, some substitutions are subtracted from the computational output to generate the library. For example the resulting output of a computational screening calculation or calculations may be filtered so that the library includes only those amino acids, or a subset of those amino acids that meet some criteria, for example that are observed at that position in an alignment of sequences. In an alternate embodiment, sequence information is used to add sequences to the computational screening output. That is to say, sequence information is used to guide the choice of additional amino acids that are added to the computational output to generate the library. For example, the output set of amino acids for a given position from a computational screening calculation may be augmented to include one or more amino acids that are observed at that position in an alignment of protein sequences. In an alternate embodiment, based on sequence alignment information, one or more amino acids may be added to or subtracted from the computational screening sequence output in order to maximize coverage or diversity. For example, additional amino acids with properties similar to those that are found in a sequence alignment may be added to the library. For example, if a position is observed to have uncharged polar amino acids in a sequence alignment, additional uncharged polar amino acids may be included in the library at that position.

[160] Libraries may be processed further to generate subsequent libraries. In this way, the output from a computational screening calculation or calculations may be thought of as a primary library. This primary library may be combined with other primary libraries from other calculations or other libraries, processed using subsequent calculations, sequence information, or other analyses, or processed experimentally to generate a subsequent library, herein referred to as a secondary library. As will be appreciated from this description, the use of sequence information to guide or filter libraries, discussed above, is itself one method of generating secondary libraries from primary libraries. Generation of secondary libraries gives the user greater control of the parameters within a library.

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This enables more efficient experimental screening, and may allow feedback from experimental results to be interpreted more easily, providing a more efficient design/experimentation cycle.

[161] There are a wide variety of methods to generate secondary libraries from primary libraries. For example, USSN 10/218,102 and PCT WO 02/25588, describes methods for secondary library generation that find use in the present invention. Typically some selection step occurs in which a primary library is processed in some way. For example, in one embodiment a selection step occurs wherein some set of primary sequences are chosen to form the secondary library. In an alternate embodiment, a selection step is a computational step, again generally including a selection step, wherein some subset of the primary library is chosen and then subjected to further computational analysis, including both further computational screening as well as techniques such as "*in silico*" shuffling or recombination (see, for example US 5,830,721; US 5,811,238; US 5,605,793; and US 5,837,458, error-prone PCR, for example using modified nucleotides; known mutagenesis techniques including the use of multi-cassettes; and DNA shuffling (Crameri *et al.*, 1998, *Nature* 391:288-291; Coco *et al.*, 2001, *Nat Biotechnol* 19:354-9; Coco *et al.*, 2002, *Nat Biotechnol*, 20:1246-50), heterogeneous DNA samples (US 5,939,250); ITCHY (Ostermeier *et al.*, 1999, *Nat Biotechnol* 17:1205-1209); StEP (Zhao *et al.*, 1998, *Nat Biotechnol* 16:258-261), GSSM (US 6,171,820 and US 5,965,408); *in vivo* homologous recombination, ligase assisted gene assembly, end-complementary PCR, profusion (Roberts & Szostak, 1997, *Proc Natl Acad Sci USA* 94:12297-12302); yeast/bacteria surface display (Lu *et al.*, 1995, *Biotechnology* 13:366-372); Seed & Aruffo, 1987, *Proc Natl Acad Sci USA* 84(10):3365-3369; Boder & Wittrup, 1997, *Nat Biotechnol* 15:553-557). In an alternate embodiment, a selection step occurs that is an experimental step, for example any of the library screening steps below, wherein some subset of the primary library is chosen and then recombined experimentally, for example using one of the directed evolution methods discussed below, to form a secondary library. In a preferred embodiment, the primary library is generated and processed as outlined in US 6,403,312.

[162] Generation of secondary and subsequent libraries finds broad use in the present invention. In one embodiment, different primary libraries may be combined to generate a secondary or subsequent library. In another embodiment, secondary libraries may be generated by sampling sequence diversity at highly mutatable or highly conserved positions. The primary library may be analyzed to determine which amino acid positions in the template protein have high mutational frequency, and which positions have low mutational frequency. For example, positions in a protein that show a great deal of mutational diversity in computational screening may be fixed in a subsequent round of design calculations. A filtered set of the same size as the first would now show diversity at positions that were largely conserved in the first library. Alternatively, the secondary library may be generated by varying the amino acids at the positions that have high numbers of mutations, while keeping constant the positions that do not have mutations above a certain frequency.

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[163] This discussion is not meant to constrain generation of libraries subsequent to primary libraries to secondary libraries. As will be appreciated, primary and secondary libraries may be processed further to generate tertiary libraries, quaternary libraries, and so on. In this way, library generation is an iterative process. For example, tertiary libraries may be constructed using a variety of additional steps applied to one or more secondary libraries; for example, further computational processing may occur, secondary libraries may be recombined, or subsets of different secondary libraries may be combined. In a preferred embodiment, a tertiary library may be generated by combining secondary libraries. For example, primary and/or secondary libraries that analyzed different parts of a protein may be combined to generate a tertiary library that treats the combined parts of the protein. In an alternate embodiment, the variants from a primary library may be combined with the variants from another primary library to provide a combined tertiary library at lower computational cost than creating a very long filtered set. These combinations may be used, for example, to analyze large proteins, especially large multi-domain proteins, of which Fc is an example. Thus the above description of secondary library generation applies to generating any library subsequent to a primary library, the end result being a final library that may screened experimentally to obtain protein variants optimized for a design goal. These examples are not meant to constrain generation of secondary libraries to any particular application or theory of operation for the present invention. Rather, these examples are meant to illustrate that generation of secondary libraries, and subsequent libraries such as tertiary libraries and so on, is broadly useful in computational screening methodology for library generation.

Experimental Production and Screening

[164] The present invention provides methods for producing and screening libraries of Fc variants. The described methods are not meant to constrain the present invention to any particular application or theory of operation. Rather, the provided methods are meant to illustrate generally that one or more Fc variants or one or more libraries of Fc variants may be produced and screened experimentally to obtain optimized Fc variants. Fc variants may be produced and screened in any context, whether as an Fc region as precisely defined herein, a domain or fragment thereof, or a larger polypeptide that comprises Fc such as an antibody or Fc fusion. General methods for antibody molecular biology, expression, purification, and screening are described in Antibody Engineering, edited by Duebel & Kontermann, Springer-Verlag, Heidelberg, 2001; and Hayhurst & Georgiou, 2001, *Curr Opin Chem Biol* 5:683-689; Maynard & Georgiou, 2000, *Annu Rev Biomed Eng* 2:339-76.

[165] In one embodiment of the present invention, the library sequences are used to create nucleic acids that encode the member sequences, and that may then be cloned into host cells, expressed and assayed, if desired. Thus, nucleic acids, and particularly DNA, may be made that encode each member protein sequence. These practices are carried out using well-known procedures. For example, a variety of methods that may find use in the present invention are described in Molecular Cloning - A Laboratory Manual, 3rd Ed. (Maniatis, Cold Spring Harbor Laboratory Press, New York,

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2001), and Current Protocols in Molecular Biology (John Wiley & Sons). As will be appreciated by those skilled in the art, the generation of exact sequences for a library comprising a large number of sequences is potentially expensive and time consuming. Accordingly, there are a variety of techniques that may be used to efficiently generate libraries of the present invention. Such methods that may find use in the present invention are described or referenced in US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 01/40091; and PCT WO 02/25588. Such methods include but are not limited to gene assembly methods, PCR-based method and methods which use variations of PCR, ligase chain reaction-based methods, pooled oligo methods such as those used in synthetic shuffling, error-prone amplification methods and methods which use oligos with random mutations, classical site-directed mutagenesis methods, cassette mutagenesis, and other amplification and gene synthesis methods. As is known in the art, there are a variety of commercially available kits and methods for gene assembly, mutagenesis, vector subcloning, and the like, and such commercial products find use in the present invention for generating nucleic acids that encode Fc variant members of a library.

[166] The Fc variants of the present invention may be produced by culturing a host cell transformed with nucleic acid, preferably an expression vector, containing nucleic acid encoding the Fc variants, under the appropriate conditions to induce or cause expression of the protein. The conditions appropriate for expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. A wide variety of appropriate host cells may be used, including but not limited to mammalian cells, bacteria, insect cells, and yeast. For example, a variety of cell lines that may find use in the present invention are described in the ATCC® cell line catalog, available from the American Type Culture Collection.

[167] In a preferred embodiment, the Fc variants are expressed in mammalian expression systems, including systems in which the expression constructs are introduced into the mammalian cells using virus such as retrovirus or adenovirus. Any mammalian cells may be used, with human, mouse, rat, hamster, and primate cells being particularly preferred. Suitable cells also include known research cells, including but not limited to Jurkat T cells, NIH3T3, CHO, COS, and 293 cells. In an alternately preferred embodiment, library proteins are expressed in bacterial cells. Bacterial expression systems are well known in the art, and include *Escherichia coli* (*E. coli*), *Bacillus subtilis*, *Streptococcus cremoris*, and *Streptococcus lividans*. In alternate embodiments, Fc variants are produced in insect cells or yeast cells. In an alternate embodiment, Fc variants are expressed *in vitro* using cell free translation systems. In vitro translation systems derived from both prokaryotic (e.g. *E. coli*) and eukaryotic (e.g. wheat germ, rabbit reticulocytes) cells are available and may be chosen based on the expression levels and functional properties of the protein of interest. For example, as appreciated by those skilled in the art, *in vitro* translation is required for some display technologies, for example ribosome display. In addition, the Fc variants may be produced by chemical synthesis methods.

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[168] The nucleic acids that encode the Fc variants of the present invention may be incorporated into an expression vector in order to express the protein. A variety of expression vectors may be utilized for protein expression. Expression vectors may comprise self-replicating extra-chromosomal vectors or vectors which integrate into a host genome. Expression vectors are constructed to be compatible with the host cell type. Thus expression vectors which find use in the present invention include but are not limited to those which enable protein expression in mammalian cells, bacteria, insect cells, yeast, and in *in vitro* systems. As is known in the art, a variety of expression vectors are available, commercially or otherwise, that may find use in the present invention for expressing Fc variant proteins.

[169] Expression vectors typically comprise a protein operably linked with control or regulatory sequences, selectable markers, any fusion partners, and/or additional elements. By "operably linked" herein is meant that the nucleic acid is placed into a functional relationship with another nucleic acid sequence. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the Fc variant, and are typically appropriate to the host cell used to express the protein. In general, the transcriptional and translational regulatory sequences may include promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. As is also known in the art, expression vectors typically contain a selection gene or marker to allow the selection of transformed host cells containing the expression vector. Selection genes are well known in the art and will vary with the host cell used.

[170] Fc variants may be operably linked to a fusion partner to enable targeting of the expressed protein, purification, screening, display, and the like. Fusion partners may be linked to the Fc variant sequence via a linker sequences. The linker sequence will generally comprise a small number of amino acids, typically less than ten, although longer linkers may also be used. Typically, linker sequences are selected to be flexible and resistant to degradation. As will be appreciated by those skilled in the art, any of a wide variety of sequences may be used as linkers. For example, a common linker sequence comprises the amino acid sequence GGGGS. A fusion partner may be a targeting or signal sequence that directs Fc variant protein and any associated fusion partners to a desired cellular location or to the extracellular media. As is known in the art, certain signaling sequences may target a protein to be either secreted into the growth media, or into the periplasmic space, located between the inner and outer membrane of the cell. A fusion partner may also be a sequence that encodes a peptide or protein that enables purification and/or screening. Such fusion partners include but are not limited to polyhistidine tags (His-tags) (for example H_6 and H_{10} or other tags for use with Immobilized Metal Affinity Chromatography (IMAC) systems (e.g. Ni^{+2} affinity columns)), GST fusions, MBP fusions, Strep-tag, the BSP biotinylation target sequence of the bacterial enzyme BirA, and epitope tags which are targeted by antibodies (for example c-myc tags, flag-tags, and the like). As will be appreciated by those skilled in the art, such tags may be useful for purification, for screening, or both.

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For example, an Fc variant may be purified using a His-tag by immobilizing it to a Ni^{+2} affinity column, and then after purification the same His-tag may be used to immobilize the antibody to a Ni^{+2} coated plate to perform an ELISA or other binding assay (as described below). A fusion partner may enable the use of a selection method to screen Fc variants (see below). Fusion partners that enable a variety of selection methods are well-known in the art, and all of these find use in the present invention. For example, by fusing the members of an Fc variant library to the gene III protein, phage display can be employed (Kay *et al.*, Phage display of peptides and proteins: a laboratory manual, Academic Press, San Diego, CA, 1996; Lowman *et al.*, 1991, *Biochemistry* 30:10832-10838; Smith, 1985, *Science* 228:1315-1317). Fusion partners may enable Fc variants to be labeled. Alternatively, a fusion partner may bind to a specific sequence on the expression vector, enabling the fusion partner and associated Fc variant to be linked covalently or noncovalently with the nucleic acid that encodes them. For example, USSN 09/642,574; USSN 10/080,376; USSN 09/792,630; USSN 10/023,208; USSN 09/792,626; USSN 10/082,671; USSN 09/953,351; USSN 10/097,100; USSN 60/366,658; PCT WO 00/22906; PCT WO 01/49058; PCT WO 02/04852; PCT WO 02/04853; PCT WO 02/08023; PCT WO 01/28702; and PCT WO 02/07466 describe such a fusion partner and technique that may find use in the present invention.

[171] The methods of introducing exogenous nucleic acid into host cells are well known in the art, and will vary with the host cell used. Techniques include but are not limited to dextran-mediated transfection, calcium phosphate precipitation, calcium chloride treatment, polybrene mediated transfection, protoplast fusion, electroporation, viral or phage infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. In the case of mammalian cells, transfection may be either transient or stable.

[172] In a preferred embodiment, Fc variant proteins are purified or isolated after expression. Proteins may be isolated or purified in a variety of ways known to those skilled in the art. Standard purification methods include chromatographic techniques, including ion exchange, hydrophobic interaction, affinity, sizing or gel filtration, and reversed-phase, carried out at atmospheric pressure or at high pressure using systems such as FPLC and HPLC. Purification methods also include electrophoretic, immunological, precipitation, dialysis, and chromatofocusing techniques. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. As is well known in the art, a variety of natural proteins bind Fc and antibodies, and these proteins can find use in the present invention for purification of Fc variants. For example, the bacterial proteins A and G bind to the Fc region. Likewise, the bacterial protein L binds to the Fab region of some antibodies, as of course does the antibody's target antigen. Purification can often be enabled by a particular fusion partner. For example, Fc variant proteins may be purified using glutathione resin if a GST fusion is employed, Ni^{+2} affinity chromatography if a His-tag is employed, or immobilized anti-flag antibody if a flag-tag is used. For general guidance in suitable purification techniques, see Protein Purification: Principles and Practice, 3rd Ed., Scopes, Springer-Verlag, NY, 1994. The degree of

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purification necessary will vary depending on the screen or use of the Fc variants. In some instances no purification is necessary. For example in one embodiment, if the Fc variants are secreted, screening may take place directly from the media. As is well known in the art, some methods of selection do not involve purification of proteins. Thus, for example, if a library of Fc variants is made into a phage display library, protein purification may not be performed.

[173] Fc variants may be screened using a variety of methods, including but not limited to those that use *in vitro* assays, *in vivo* and cell-based assays, and selection technologies. Automation and high-throughput screening technologies may be utilized in the screening procedures. Screening may employ the use of a fusion partner or label. The use of fusion partners has been discussed above. By "labeled" herein is meant that the Fc variants of the invention have one or more elements, isotopes, or chemical compounds attached to enable the detection in a screen. In general, labels fall into three classes: a) immune labels, which may be an epitope incorporated as a fusion partner that is recognized by an antibody, b) isotopic labels, which may be radioactive or heavy isotopes, and c) small molecule labels, which may include fluorescent and colorimetric dyes, or molecules such as biotin that enable other labeling methods. Labels may be incorporated into the compound at any position and may be incorporated *in vitro* or *in vivo* during protein expression.

[174] In a preferred embodiment, the functional and/or biophysical properties of Fc variants are screened in an *in vitro* assay. *In vitro* assays may allow a broad dynamic range for screening properties of interest. Properties of Fc variants that may be screened include but are not limited to stability, solubility, and affinity for Fc ligands, for example Fc□Rs. Multiple properties may be screened simultaneously or individually. Proteins may be purified or unpurified, depending on the requirements of the assay. In one embodiment, the screen is a qualitative or quantitative binding assay for binding of Fc variants to a protein or nonprotein molecule that is known or thought to bind the Fc variant. In a preferred embodiment, the screen is a binding assay for measuring binding to the antibody's or Fc fusions' target antigen. In an alternately preferred embodiment, the screen is an assay for binding of Fc variants to an Fc ligand, including but are not limited to the family of Fc□Rs, the neonatal receptor FcRn, the complement protein C1q, and the bacterial proteins A and G. Said Fc ligands may be from any organism, with humans, mice, rats, rabbits, and monkeys preferred. Binding assays can be carried out using a variety of methods known in the art, including but not limited to FRET (Fluorescence Resonance Energy Transfer) and BRET (Bioluminescence Resonance Energy Transfer) -based assays, AlphaScreen™ (Amplified Luminescent Proximity Homogeneous Assay), Scintillation Proximity Assay, ELISA (Enzyme-Linked Immunosorbent Assay), SPR (Surface Plasmon Resonance, also known as BIACORE®), isothermal titration calorimetry, differential scanning calorimetry, gel electrophoresis, and chromatography including gel filtration. These and other methods may take advantage of some fusion partner or label of the Fc variant. Assays may employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels.

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[175] The biophysical properties of Fc variant proteins, for example stability and solubility, may be screened using a variety of methods known in the art. Protein stability may be determined by measuring the thermodynamic equilibrium between folded and unfolded states. For example, Fc variant proteins of the present invention may be unfolded using chemical denaturant, heat, or pH, and this transition may be monitored using methods including but not limited to circular dichroism spectroscopy, fluorescence spectroscopy, absorbance spectroscopy, NMR spectroscopy, calorimetry, and proteolysis. As will be appreciated by those skilled in the art, the kinetic parameters of the folding and unfolding transitions may also be monitored using these and other techniques. The solubility and overall structural integrity of an Fc variant protein may be quantitatively or qualitatively determined using a wide range of methods that are known in the art. Methods which may find use in the present invention for characterizing the biophysical properties of Fc variant proteins include gel electrophoresis, chromatography such as size exclusion chromatography and reversed-phase high performance liquid chromatography, mass spectrometry, ultraviolet absorbance spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, differential scanning calorimetry, analytical ultra-centrifugation, dynamic light scattering, proteolysis, and cross-linking, turbidity measurement, filter retardation assays, immunological assays, fluorescent dye binding assays, protein-staining assays, microscopy, and detection of aggregates via ELISA or other binding assay. Structural analysis employing X-ray crystallographic techniques and NMR spectroscopy may also find use. In one embodiment, stability and/or solubility may be measured by determining the amount of protein solution after some defined period of time. In this assay, the protein may or may not be exposed to some extreme condition, for example elevated temperature, low pH, or the presence of denaturant. Because function typically requires a stable, soluble, and/or well-folded/structured protein, the aforementioned functional and binding assays also provide ways to perform such a measurement. For example, a solution comprising an Fc variant could be assayed for its ability to bind target antigen, then exposed to elevated temperature for one or more defined periods of time, then assayed for antigen binding again. Because unfolded and aggregated protein is not expected to be capable of binding antigen, the amount of activity remaining provides a measure of the Fc variant's stability and solubility.

[176] In a preferred embodiment, the library is screened using one or more cell-based or *in vivo* assays. For such assays, Fc variant proteins, purified or unpurified, are typically added exogenously such that cells are exposed to individual variants or pools of variants belonging to a library. These assays are typically, but not always, based on the function of an antibody or Fc fusion that comprises the Fc variant; that is, the ability of the antibody or Fc fusion to bind a target antigen and mediate some biochemical event, for example effector function, ligand/receptor binding inhibition, apoptosis, and the like. Such assays often involve monitoring the response of cells to antibody or Fc fusion, for example cell survival, cell death, change in cellular morphology, or transcriptional activation such as cellular expression of a natural gene or reporter gene. For example, such assays may measure the

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ability of Fc variants to elicit ADCC, ADCP, or CDC. For some assays additional cells or components, that is in addition to the target cells, may need to be added, for example example serum complement, or effector cells such as peripheral blood monocytes (PBMCs), NK cells, macrophages, and the like. Such additional cells may be from any organism, preferably humans, mice, rat, rabbit, and monkey. Antibodies and Fc fusions may cause apoptosis of certain cell lines expressing the antibody's target antigen, or they may mediate attack on target cells by immune cells which have been added to the assay. Methods for monitoring cell death or viability are known in the art, and include the use of dyes, immunochemical, cytochemical, and radioactive reagents. For example, caspase staining assays may enable apoptosis to be measured, and uptake or release of radioactive substrates or fluorescent dyes such as alamar blue may enable cell growth or activation to be monitored. In a preferred embodiment, the DELFIA® EuTDA-based cytotoxicity assay (Perkin Elmer, MA) is used. Alternatively, dead or damaged target cells may be monitoried by measuring the release of one or more natural intracellular proteins, for example lactate dehydrogenase. Transcriptional activation may also serve as a method for assaying function in cell-based assays. In this case, response may be monitored by assaying for natural genes or proteins which may be upregulated, for example the release of certain interleukins may be measured, or alternatively readout may be via a reporter construct. Cell-based assays may also involve the measure of morphological changes of cells as a response to the presence of an Fc variant. Cell types for such assays may be prokaryotic or eukaryotic, and a variety of cell lines that are known in the art may be employed.

[177] Alternatively, cell-based screens are performed using cells that have been transformed or transfected with nucleic acids encoding the Fc variants. That is, Fc variant proteins are not added exogenously to the cells. For example, in one embodiment, the cell-based screen utilizes cell surface display. A fusion partner can be employed that enables display of Fc variants on the surface of cells (Witrrup, 2001, *Curr Opin Biotechnol*, 12:395-399). Cell surface display methods that may find use in the present invention include but are not limited to display on bacteria (Georgiou *et al.*, 1997, *Nat Biotechnol* 15:29-34; Georgiou *et al.*, 1993, *Trends Biotechnol* 11:6-10; Lee *et al.*, 2000, *Nat Biotechnol* 18:645-648; Jun *et al.*, 1998, *Nat Biotechnol* 16:576-80), yeast (Boder & Wittrup, 2000, *Methods Enzymol* 328:430-44; Boder & Wittrup, 1997, *Nat Biotechnol* 15:553-557), and mammalian cells (Whitehorn *et al.*, 1995, *Bio/technology* 13:1215-1219). In an alternate embodiment, Fc variant proteins are not displayed on the surface of cells, but rather are screened intracellularly or in some other cellular compartment. For example, periplasmic expression and cytometric screening (Chen *et al.*, 2001, *Nat Biotechnol* 19: 537-542), the protein fragment complementation assay (Johnsson & Varshavsky, 1994, *Proc Natl Acad Sci USA* 91:10340-10344.; Pelletier *et al.*, 1998, *Proc Natl Acad Sci USA* 95:12141-12146), and the yeast two hybrid screen (Fields & Song, 1989, Nature 340:245-246) may find use in the present invention. Alternatively, if a polypeptide that comprises the Fc variants, for example an antibody or Fc fusion, imparts some selectable growth advantage to a cell, this property may be used to screen or select for Fc variants.

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[178] As is known in the art, a subset of screening methods are those that select for favorable members of a library. Said methods are herein referred to as "selection methods", and these methods find use in the present invention for screening Fc variant libraries. When libraries are screened using a selection method, only those members of a library that are favorable, that is which meet some selection criteria, are propagated, isolated, and/or observed. As will be appreciated, because only the most fit variants are observed, such methods enable the screening of libraries that are larger than those screenable by methods that assay the fitness of library members individually. Selection is enabled by any method, technique, or fusion partner that links, covalently or noncovalently, the phenotype of an Fc variant with its genotype, that is the function of an Fc variant with the nucleic acid that encodes it. For example the use of phage display as a selection method is enabled by the fusion of library members to the gene III protein. In this way, selection or isolation of variant proteins that meet some criteria, for example binding affinity for an FcγR, also selects for or isolates the nucleic acid that encodes it. Once isolated, the gene or genes encoding Fc variants may then be amplified. This process of isolation and amplification, referred to as panning, may be repeated, allowing favorable Fc variants in the library to be enriched. Nucleic acid sequencing of the attached nucleic acid ultimately allows for gene identification.

[179] A variety of selection methods are known in the art that may find use in the present invention for screening Fc variant libraries. These include but are not limited to phage display (Phage display of peptides and proteins: a laboratory manual, Kay *et al.*, 1996, Academic Press, San Diego, CA, 1996; Lowman *et al.*, 1991, *Biochemistry* 30:10832-10838; Smith, 1985, *Science* 228:1315-1317) and its derivatives such as selective phage infection (Malmborg *et al.*, 1997, J Mol Biol 273:544-551), selectively infective phage (Krebber *et al.*, 1997, *J Mol Biol* 268:619-630), and delayed infectivity panning (Benhar *et al.*, 2000, *J Mol Biol* 301:893-904), cell surface display (Witrrup, 2001, *Curr Opin Biotechnol*, 12:395-399) such as display on bacteria (Georgiou *et al.*, 1997, *Nat Biotechnol* 15:29-34; Georgiou *et al.*, 1993, *Trends Biotechnol* 11:6-10; Lee *et al.*, 2000, *Nat Biotechnol* 18:645-648; Jun *et al.*, 1998, *Nat Biotechnol* 16:576-80), yeast (Boder & Wittrup, 2000, *Methods Enzymol* 328:430-44; Boder & Wittrup, 1997, *Nat Biotechnol* 15:553-557), and mammalian cells (Whitehorn *et al.*, 1995, *Bio/technology* 13:1215-1219), as well as *in vitro* display technologies (Amstutz *et al.*, 2001, *Curr Opin Biotechnol* 12:400-405) such as polysome display (Mattheakis *et al.*, 1994, *Proc Natl Acad Sci USA* 91:9022-9026), ribosome display (Hanes *et al.*, 1997, *Proc Nati Acad Sci USA* 94:4937-4942), mRNA display (Roberts & Szostak, 1997, *Proc Natl Acad Sci USA 94*:12297-12302; Nemoto *et al.*, 1997, *FEBS Lett* 414:405-408), and ribosome-inactivation display system (Zhou *et al.*, 2002, *J Am Chem Soc* 124, 538-543)

[180] Other selection methods that may find use in the present invention include methods that do not rely on display, such as *in vivo* methods including but not limited to periplasmic expression and cytometric screening (Chen *et al.*, 2001, *Nat Biotechnol* 19:537-542), the protein fragment complementation assay (Johnsson & Varshavsky, 1994, *Proc Natl Acad Sci USA* 91:10340-10344;

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Pelletier *et al.*, 1998, *Proc Natl Acad Sci USA* 95:12141-12146), and the yeast two hybrid screen (Fields & Song, 1989, *Nature* 340:245-246) used in selection mode (Visintin *et al.*, 1999, *Proc Natl Acad Sci USA* 96:11723-11728). In an alternate embodiment, selection is enabled by a fusion partner that binds to a specific sequence on the expression vector, thus linking covalently or noncovalently the fusion partner and associated Fc variant library member with the nucleic acid that encodes them. For example, USSN 09/642,574; USSN 10/080,376; USSN 09/792,630; USSN 10/023,208; USSN 09/792,626; USSN 10/082,671; USSN 09/953,351; USSN 10/097,100; USSN 60/366,658; PCT WO 00/22906; PCT WO 01/49058; PCT WO 02/04852; PCT WO 02/04853; PCT WO 02/08023; PCT WO 01/28702; and PCT WO 02/07466 describe such a fusion partner and technique that may find use in the present invention. In an alternative embodiment, *in vivo* selection can occur if expression of a polypeptide that comprises the Fc variant, such as an antibody or Fc fusion, imparts some growth, reproduction, or survival advantage to the cell.

[181] A subset of selection methods referred to as "directed evolution" methods are those that include the mating or breading of favorable sequences during selection, sometimes with the incorporation of new mutations. As will be appreciated by those skilled in the art, directed evolution methods can facilitate identification of the most favorable sequences in a library, and can increase the diversity of sequences that are screened. A variety of directed evolution methods are known in the art that may find use in the present invention for screening Fc variant libraries, including but not limited to DNA shuffling (PCT WO 00/42561 A3; PCT WO 01/70947 A3), exon shuffling (US 6,365,377; Kolkman & Stemmer, 2001, *Nat Biotechnol* 19:423-428), family shuffling (Crameri *et al.*, 1998, *Nature* 391:288-291; US 6,376,246), RACHITT™ (Coco *et al.*, 2001, *Nat Biotechnol* 19:354- 359; PCT WO 02/06469), STEP and random priming of *in vitro* recombination (Zhao *et al.*, 1998, *Nat Biotechnol* 16:258-261; Shao *et al.*, 1998, *Nucleic Acids Res* 26:681-683), exonuclease mediated gene assembly (US 6,352,842; US 6,361,974), Gene Site Saturation Mutagenesis™ (US 6,358,709), Gene Reassembly™ (US 6,358,709), SCRATCHY (Lutz et al., 2001, *Proc Natl Acad Sci USA* 98:11248-11253), DNA fragmentation methods (Kikuchi *et al.*, *Gene* 236:159-167), single-stranded DNA shuffling (Kikuchi *et al.*, 2000, *Gene* 243:133-137), and AMEsystem™ directed evolution protein engineering technology (Applied Molecular Evolution) (US US 5,824,514; US 5,817,483; US 5,814,476; US 5,763,192; US 5,723,323).

[182] The biological properties of the antibodies and Fc fusions that comprise the Fc variants of the present invention may be characterized in cell, tissue, and whole organism experiments. As is know in the art, drugs are often tested in animals, including but not limited to mice, rats, rabbits, dogs, cats, pigs, and monkeys, in order to measure a drug's efficacy for treatment against a disease or disease model, or to measure a drug's pharmacokinetics, toxicity, and other properties. Said animals may be referred to as disease models. Therapeutics are often tested in mice, including but not limited to nude mice, SCID mice, xenograft mice, and transgenic mice (including knockins and knockouts). For example, an antibody or Fc fusion of the present invention that is intended as an anti-cancer

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therapeutic may be tested in a mouse cancer model, for example a xenograft mouse. In this method, a tumor or tumor cell line is grafted onto or injected into a mouse, and subsequently the mouse is treated with the therapeutic to determine the ability of the antibody or Fc fusion to reduce or inhibit cancer growth. Such experimentation may provide meaningful data for determination of the potential of said antibody or Fc fusion to be used as a therapeutic. Any organism, preferably mammals, may be used for testing. For example because of their genetic similarity to humans, monkeys can be suitable therapeutic models, and thus may be used to test the efficacy, toxicity, pharmacokinetics, or other property of the antibodies and Fc fusions of the present invention. Tests of the antibodies and Fc fusions of the present invention in humans are ultimately required for approval as drugs, and thus of course these experiments are contemplated. Thus the antibodies and Fc fusions of the present invention may be tested in humans to determine their therapeutic efficacy, toxicity, pharmacokinetics, and/or other clinical properties.

EXAMPLES

[183] Examples are provided below to illustrate the present invention. These examples are not meant to constrain the present invention to any particular application or theory of operation.

[184] For all positions discussed in the present invention, numbering is according to the EU index as in Kabat (Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest,* 5th Ed., United States Public Health Svice, National Institutes of Health, Bethesda). Those skilled in the art of antibodies will appreciate that this convention consists of nonsequential numbering in specific regions of an immunoglobulin sequence, enabling a normalized reference to conserved positions in immunoglobulin families. Accordingly, the positions of any given immunoglobulin as defined by the EU index will not necessarily correspond to its sequential sequence. Figure 3 shows the sequential and EU index numbering schemes for the antibody alemtuzumab in order to illustrate this principal more clearly. It should also be noted that polymorphisms have been observed at a number of Fc positions, including but not limited to Kabat 270, 272, 312, 315, 356, and 358, and thus slight differences between the presented sequence and sequences in the scientific literature may exist.

[185] Example 1. Computational Screening and Design of Fc Libraries

Computational screening calculations were carried out to design optimized Fc variants. Fc variants were computationally screened, constructed, and experimentally investigated over several computation/experimention cycles. For each successive cycle, experimental data provided feedback into the next set of computational screening calculations and library design. All computational screening calculations and library design are presented in Example 1. For each set of calculations, a table is provided that presents the results and provides relevant information and parameters.

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[186] Several different structures of Fc bound bound to the extracellular domain of Fc□Rs served as template structures for the computational screening calculations. Publicly available Fc/FcγR complex structures included pdb accession code 1E4K (Sondermann *et al.*, 2000, *Nature* 406:267-273.), and pdb accession codes 1IIS and 1IIX (Radaev *et al.*, 2001, *J Biol Chem* 276:16469-16477). The extracellular regions of Fc□RIIIb and FcγRIIIa are 96% identical, and therefore the use of the Fc/FcγRIIIb structure is essentially equivalent to use of FcγRIIIa. Nonetheless, for some calculations, a more precise Fc/Fc□RIIIa template structure was constructed by modeling a D129G mutation in the 1IIS and 1E4K structures (referred to as D129G 1IIS and D129G 1E4K template structures). In addition, the structures for human Fc bound to the extracellular domains of human FcγRIIb, human F158 Fc□RIIIa, and mouse FcγRIII were modeled using standard methods, the available Fc□R sequence information, the aforementioned Fc/FcγR structures, as well as structural information for unbound complexes (pdb accession code 1H9V)(Sondermann *et al.*, 2001, *J Mol Biol* 309:737-749) (pdb accession code 1FCG)(Maxwell *et al.*, 1999, *Nat Struct Biol* 6:437-442), Fc□RIIb (pdb accession code 2FCB)(Sondermann *et al.*, 1999, *Embo J* 18:1095-1103), and Fc□RIIIb (pdb accession code 1E4J)(Sondermann *et al.*, 2000, *Nature* 406:267-273.).

[187] Variable positions and amino acids to be considered at those positions were chosen by visual inspection of the aforementioned Fc/FcγR and FcγR structures, and using solvent accessibility information and sequence information. Sequence information of Fcs and FcγRs was particularly useful for determining variable positions at which substitutions may provide distinguishing affinities between activating and inhibitory receptors. Virtually all Cγ2 positions were screened computationally. The Fc structure is a homodimer of two heavy chains (labeled chains A and B in the 1IIS, 1IIX, and 1E4K structures) that each include the hinge and C□2-C□3 domains (shown in Figure 2). Because the Fc□R (labeled chain C in the 1IIS, 1IIX, and 1E4K structures) binds asymmetrically to the Fc homodimer, each chain was often considered separately in design calculations. For some calculations, Fc and/or Fc□R residues proximal to variable position residues were floated, that is the amino acid conformation but not the amino acid identity was allowed to vary in a protein design calculation to allow for conformational adjustments. These are indicated below the table for each set of calculations when relevant. Considered amino acids typically belonged to either the Core, Core XM, Surface, Boundary, Boundary XM, or All 20 classifications, unless noted otherwise. These classifications are defined as follows: Core = alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine; Core XM = alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, and tryptophan; Surface = alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine and histidine; Boundary = alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine, histidine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine; Boundary XM = Boundary = alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine, histidine, valine, isoleucine, leucine, phenylalanine, tyrosine, and tryptophan; All 20 = all 20 naturally occurring amino acids.

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[188] The majority of calculations followed one of two general types of computational screening methods. In one method, the conformations of amino acids at variable positions were represented as a set of backbone-independent side chain rotamers derived from the rotamer library of Dunbrack & Cohen (Dunbrack *et al.*, 1997, *Protein Sci* 6:1661-1681). The energies of all possible combinations of the considered amino acids at the chosen variable positions were calculated using a force field containing terms describing van der Waals, solvation, electrostatic, and hydrogen bond interactions, and the optimal (ground state) sequence was determined using a Dead End Elimination (DEE) algorithm. As will be appreciated by those in the art, the predicted lowest energy sequence is not necessarily the true lowest energy sequence because of errors primarily in the scoring function, coupled with the fact that subtle conformational differences in proteins can result in dramatic differences in stability. However, the predicted ground state sequence is likely to be close to the true ground state, and thus additional favorable diversity can be explored by evaluating the energy of sequences that are close in sequence space and energy around the predicted ground state. To accomplish this, as well as to generate a diversity of sequences for a library, a Monte Carlo (MC) algorithm was used to evaluate the energies of 1000 similar sequences around the predicted ground state. The number of sequences out of the 1000 sequence set that contain that amino acid at that variable position is referred to as the occupancy for that substitution, and this value may reflect how favorable that substitution is. This computational screening method is substantially similar to Protein Design Automation® (PDA®) technology, as described in US 6,188,965; US 6,269,312; US 6,403,312; USSN 09/782,004; USSN 09/927,790; USSN 10/218,102; PCT WO 98/07254; PCT WO 01/40091; and PCT WO 02/25588, and for ease of description, is referred to as PDA® technology throughout the examples. Tables that present the results of these calculations provide for each variable position on the designated chain (column 1) the amino acids considered at each variable position (column 2), the WT Fc amino acid identity at each variable position (column 3), the amino acid identity at each variable position in the DEE ground state sequence (column 4), and the set of amino acids and corresponding occupancy that are observed in the Monte Carlo output (column 5).

[189] Other calculations utilized a genetic algorithm (GA) to screen for low energy sequences, with energies being calculated during each round of "evolution" for those sequences being sampled. The conformations of amino acids at variable and floated positions were represented as a set of side chain rotamers derived from a backbone-independent rotamer library using a flexible rotamer model (Mendes *et al.*, 1999, *Proteins* 37:530-543). Energies were calculated using a force field containing terms describing van der Waals, solvation, electrostatic, and hydrogen bond interactions. This calculation generated a list of 300 sequences which are predicted to be low in energy. To facilitate analysis of the results and library generation, the 300 output sequences were clustered computationally into 10 groups of similar sequences using a nearest neighbor single linkage hierarchical clustering algorithm to assign sequences to related groups based on similarity scores (Diamond, 1995, *Acta Cryst* D51:127-135). That is, all sequences within a group are most similar to

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all other sequences within the same group and less similar to sequences in other groups. The lowest energy sequence from each of these ten clusters are used as a representative of each group, and are presented as results. This computational screening method is substantially similar to Sequence Prediction Algorithm™ (SPA™) technology, as described in (Raha *et al.*, 2000, *Protein Sci* 9:1106-1119); USSN 09/877,695; and USSN 10/071,859, and for ease of description, is referred to as SPA™ technology throughout the examples.

[190] Computational screening was applied to design energetically favorable interactions at the Fc/Fc□R interface at groups of variable positions that mediate or potentially mediate binding with Fc□R. Because the binding interface involves a large number of Fc residues on the two different chains, and because Fc□Rs bind asymmetrically to Fc, residues were grouped in different sets of interacting variable positions, and designed in separate sets of calculations. In many cases these sets were chosen as groups of residues that were deemed to be coupled, that is the energy of one or more residues is dependent on the identity of one or more other residues. Various template structures were used, and in many cases calculations explored substitutions on both chains. For many of the variable position sets, calculations were carried out using both the PDA® and SPA™ technology computational screening methods described. The results of these calculations and relevant parameters and information are presented in Tables 1 – 30 below.

[191] Tables that present the results of these calculations provide for each variable position on the designated chain (column 1) the amino acids considered at each variable position (column 2), the WT Fc amino acid identity at each variable position (column 3), and the amino acid identity at the variable positions for the lowest energy sequence from each cluster group (columns 4-13). Tables 1-59 are broken down into two sets, as labeled below, PDA® and SPA™ technology. Column 4 of the PDA® tables show the frequency of each residue that occurs in the top 1000 sequences during that PDA® run. Thus, in the first row of Table 1, at position 328, when run using boundary amino acids as the set of variable residues for that position, L occurred 330 times in the top 1000 sequence, M occurred 302 times, etc.

[192] In addition, included within the compositions of the invention are antibodies that have any of the listed amino acid residues in the listed positions, either alone or in any combination (note preferred combinations are listed in the claims, the summary and the figures). One preferred combination is the listed amino acids residues in the listed positions in a ground state (sometimes referred to herein as the "global solution", as distinguished from the wild-type). Similarly, residue positions and particular amino acids at those residue positions may be combined between tables.

[193] For SPA™ technology tables, such as Table 4, column 4 is a SPA™ run that results in a protein with the six listed amino acids at the six listed positions (e.g. column 4 is a single protein with

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a wild-type sequence except for 239E, 265G, 267S, 269Y, 270T and 299S. Thus, each of these individual proteins are included within the invention. In addition, combinations between SPA™ proteins, both within tables and between tables, are also included.

[194] In addition, each table shows the presence or absence of carbohydrate, but specifically included are the reverse sequences; e.g. Table 1 is listed for an aglycosylated variant, but these same amino acid changes can be done on a glycosylated variant.

[195] Furthermore, each table lists the template structure used, as well as "floated" residues; for example, Table 2 used a PDA® run that floated C120, C132 and C134.

Table 1

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
328 A	Boundary	L	L	L:330 M:302 E:111 K:62 A:45 Q:39 D:36 S:30 T:28 N:10 R:7
332 A	Surface	I	R	R:247 K:209 Q:130 H:95 E:92 T:59 D:51 N:51 S:42 A:24
328 B	Boundary	L	L	L:321 M:237 T:166 K:73 R:72 S:55 Q:20 D:17 E:13 A:12 V:10 N:4
332 B	Surface	I	E	E:269 Q:180 R:145 K:111 D:97 T:78 N:65 S:28 A:14 H:13

PDA® technology, 1IIS template structure; - carbohydrate

Table 2

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Surface	S	K	E:349 D:203 K:196 A:95 Q:83 S:63 N:10 R:1
265 A	Boundary XM	D	D	D:616 N:113 L:110 E:104 S:25 A:23 Q:9
299 A	Boundary XM	T	I	I:669 H:196 V:135
327 A	Boundary XM	A	S	A:518 S:389 N:67 D:26
265 B	Boundary XM	D	Q	Q:314 R:247 N:118 I:115 A:63 E:55 D:34 S:22 K:21 V:11

PDA® technology; 1IIS template structure; + carbohydrate; floated 120 C, 132 C, 134 C

Table 3

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Surface	S	E	E:872 Q:69 D:39 K:16 A:4
265 A	Boundary XM	D	Y	Y:693 H:111 E:69 D:62 F:29 K:19 R:14 W:2 Q:1
267 A	Boundary XM	S	S	S:991 A:9
269 A	Core XM	E	F	F:938 E:59 Y:3
270 A	Surface	D	E	E:267 T:218 K:186 D:89 Q:88 R:46 S:34 N:29 H:23 A:20
299 A	Boundary XM	T	H	H:486 T:245 K:130 E:40 S:39 D:27 Q:27 A:4 N:2

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PDA® technology; 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 132 C, 133 C, 134 C

Table 4

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	Surface	S	E	Q	Q	Q	E	E	E	Q	E	E
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
267 A	All 20	S	S	S	S	S	S	S	S	S	S	S
269 A	Core	E	Y	Y	A	A	V	Y	A	A	A	A
270 A	Surface	D	T	S	A	S	T	T	T	A	A	A
299 A	All 20	T	S	S	S	S	S	S	S	S	S	S

SPA™ technology; 1IIS template structure; + carbohydrate; floated 120 C, 122 C, 132 C, 133 C, 134 C

Table 5

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
235 A	Boundary XM	L	T	T:195 V:131 L:112 W:107 K:85 F:66 Y:56 E:52 Q:38 S:37 I:34 R:29 H:26 N:23 D:9
296 A	Surface	Y	N	N:322 D:181 R:172 K:76 Y:70 Q:59 E:48 S:40 H:20 T:11 A:1
298 A	Surface	S	T	T:370 R:343 K:193 A:55 S:39
235 B	Boundary XM	L	L	L:922 I:78

PDA® technology; 1IIS template structure; - carbohydrate; floated 119 C, 128 C, 157 C

Table 6

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
235 A	All 20	L	S	S	P	S	S	S	S	S	S	S
296 A	Surface	Y	Q	Q	Q	E	E	Q	E	Q	Q	N
298 A	Surface	S	S	K	K	K	K	S	S	S	K	S
235 B	All 20	L	K	K	K	L	L	L	L	L	L	K

SPA™ technology; 1IIS template structure; + carbohydrate; floated 119 C, 128 C, 157 C

Table 7

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Surface	S	E	K:402 E:282 H:116 T:67 R:47 Q:39 D:26 A:11 S:7 N:3
265 B	Boundary XM	D	W	Y:341 W:283 I:236 V:77 F:36 H:9 T:7 E:4 K:4 A:2 D:1
327 B	Boundary XM	A	R	R:838 K: 86 H:35 E:12 T:10 Q:7 A:6 D:3 N:3
328 B	Core XM	L	L	L:1000
329 B	Core XM	P	P	P:801 A:199
330 B	Core XM	A	Y	Y:918 F:42 L:22 A:18
332 B	Surface	I	I	I:792 E:202 Q:5 K:1

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PDA® technology; 1IIS template structure; - carbohydrate; floated 88 C, 90 C, 113 C, 114 C, 116 C, 160 C, 161 C

Table 8

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	Surface	S	D	T	E	E	E	E	E	E	E	E
265 B	All 20	D	G	G	K	G	K	G	G	K	K	G
327 B	All 20	A	K	M	L	L	N	L	K	L	L	L
328 B	Core	L	M	M	M	L	A	M	L	M	L	L
329 B	Core	P	P	P	P	P	P	P	P	P	P	P
330 B	Core	A	L	A	A	A	A	A	A	A	A	A
332 B	Surface	I	I	Q	I	I	Q	Q	E	D	I	I

SPA™ technology; 1IIS template structure; + carbohydrate; floated 88 C, 90 C, 113 C, 114 C, 116 C, 160 C, 161 C

Table 9

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	Surface	S	Q	Q	Q	E	Q	E	Q	E	Q	Q
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
299 A	All 20	T	S	S	A	S	S	S	S	S	S	S
327 A	All 20	A	A	S	S	S	S	S	S	S	A	S
265 B	All 20	D	N	G	G	G	G	G	G	G	G	G

SPA™ technology; 1IIS template structure; - carbohydrate; floated 120 C, 132 C, 134 C

Table 10

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
234 A	Boundary XM	L	K	Y:401 L:260 F:151 I:82 K:63 H:17 Q:11 W:7 R:3 T:2 E:2 V:1
235 A	Boundary XM	L	L	W:777 L:200 K:12 Y:5 I:3 F:2 V:1
234 B	Boundary XM	L	W	W:427 Y:203 L:143 F:74 I:59 E:32 K:23 V:14 D:10 T:7 H:4 R:4
235 B	Boundary XM	L	W	W:380 Y:380 F:135 K:38 L:26 E:15 Q:12 H:8 R:4 T:2

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 113 C, 116 C, 132 C, 155 C, 157 C

Table 11

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
234 A	All 20	L	G	G	G	G	G	G	G	G	G	G
235 A	All 20	L	T	L	L	L	L	L	L	L	T	L
234 B	All 20	L	G	G	G	G	G	G	G	G	G	G
235 B	All 20	L	S	A	S	A	A	S	S	S	A	A

SPA™ technology; D129G 1E4K template structure; + carbohydrate; floated 113 C, 116 C, 132 C, 155 C, 157 C

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Table 12

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Boundary XM	S	E	E:235 S:122 D:94 Q:93 A:74 K:70 L:67 T:63 N:57 R:51 I:29 V:18 W:15 H:12
328 A	Boundary XM	L	L	L:688 E:121 K:43 Q:41 A:33 D:26 S:14 T:14 N:12 R:8
332 A	Boundary XM	I	W	I:155 W:95 L:82 K:79 E:74 Q:69 H:67 V:63 R:57 T:57 D:45 S:43 N:42 A:35 F:19 Y:18

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 120 C

Table 13

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	All 20	S	L	E	E	Q	E	E	K	K	K	K
328 A	All 20	L	L	Q	L	Q	K	L	L	Q	K	L
332 A	All 20	I	K	K	L	Q	A	K	L	Q	A	Q

SPA™ technology; D129G 1IIS template structure; + carbohydrate; floated 120 C

Table 14

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Boundary XM	S	I	R:195 I:169 L:126 V:91 K:89 E:61 H:52 T:50 Q:42 N:35 S:34 D:30 A:26
328 B	Boundary XM	L	L	L:671 T:165 K:40 S:38 E:28 R:17 Q:17 V:11 A:8 D:5
332 B	Boundary XM	I	I	I:387 E:157 L:151 V:78 Q:63 K:50 R:33 T:29 D:25 A:12 N:8 S:6 W:1

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 90 C, 160 C, 161 C

Table 15

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	T	L	L	L	L	L	L	L	L	L
328 B	All 20	L	M	R	M	D	T	M	L	Q	D	L
332 B	All 20	I	I	D	Q	Q	Q	L	L	T	Q	L

SPA™ technology; D129G 1IIS template structure; + carbohydrate; floated 90 C, 160 C, 161 C

Table 16

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Boundary XM	S	T	T:164 S:159 L:156 E:86 W:76 K:71 D:65 A:52 R:43 H:38 Q:38 N:31 I:14 V:7
328 B	Boundary XM	L	L	L:556 E:114 T:84 K:80 S:69 Q:36 A:31 D:15 R:11 N:4
332 B	Boundary XM	I	W	I:188 W:177 E:97 L:94 T:59 Q:57 V:54 K:52 F:51 D:34 H:33 S:27 R:26 N:18 A:17 Y:16

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 117 C

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Table 17

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	P	S	P	E	L	L	L	L	L	L
328 B	All 20	L	K	K	K	K	K	L	K	K	K	L
332 B	All 20	I	S	S	E	L	L	L	E	L	L	L

SPA™ technology; D129G 1E4K template structure; + carbohydrate; floated 117 C

Table 18

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Boundary XM	S	L	K:196 L:171 I:146 E:88 V:76 R:75 T:50 H:45 D:43 Q:39 S:30 N:22 A:19
328 A	Boundary XM	L	W	L:517 F:230 W:164 H:40 K:29 E:11 R:5 T:4
332 A	Boundary XM	I	E	I:283 L:217 E:178 Q:81 V:64 D:47 T:35 K:27 W:18 R:12 A:10 Y:7 N:7 F:6 S:5 H:3

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 87 C, 157 C, 158 C

Table 19

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	All 20	S	F	Q	E	T	P	P	T	P	P	P
328 A	All 20	L	K	R	R	K	K	M	R	K	M	R
332 A	All 20	I	L	L	I	I	E	I	E	E	I	I

SPA™ technology; D129G 1E4K template structure; + carbohydrate atoms; floated 87 C, 157 C, 158 C

Table 20

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
240 A	Core + Thr	V	V	V:698 M:162 T:140
263 A	Core + Thr	V	V	V:966 T:34
266 A	Core + Thr	V	V	V:983 T:17
325 A	Boundary	N	N	N:943 T:40 A:17
328 A	Boundary	L	L	L:610 M:363 K:27
332 A	Glu	I	E	E:1000

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 273 A, 275 A, 302 A, 323 A, 134 C

Table 21

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
240 A	All 20	V	V	A	V	V	V	V	V	V	V	V
263 A	All 20	V	V	V	V	V	V	V	V	V	V	V
266 A	All 20	V	I	V	I	I	T	V	V	V	V	I
325 A	All 20	N	A	N	N	N	Q	T	T	Q	N	T

328 A	All 20	L	K	K	L	K	L	K	L	L	L	L
332 A	Glu	I	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1IIS template structure; + carbohydrate; floated 273 A, 275 A, 302 A, 323 A, 134 C

Table 22

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
240 B	Core + Thr	V	V	V:713 T:287
263 B	Core + Thr	V	V	V:992 T:8
266 B	Core + Thr	V	V	V:976 T:24
325 B	Boundary	N	N	N:453 T:296 A:116 D:96 S:30 V:9
328 B	Boundary	L	L	L:623 M:194 T:100 R:72 K:11
332 B	Glu	I	E	E:1000

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 273 B, 275 B, 302 B, 323 B, 161 C

Table 23

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
240 B	All 20	V	A	T	A	T	T	A	A	T	T	T
263 B	All 20	V	V	A	A	T	T	V	V	T	A	T
266 B	All 20	V	V	V	V	V	V	V	V	V	I	V
325 B	All 20	N	N	K	K	N	K	K	N	N	N	N
328 B	All 20	L	R	L	L	L	L	L	L	L	L	L
332 A	Glu	I	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1IIS template structure; + carbohydrate; floated 273 B, 275 B, 302 B, 323 B, 161 C

Table 24

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
240 B	Core + Thr	V	M	V:715 M:271 T:12 I:2
263 B	Core + Thr	V	V	V:992 T:8
266 B	Core + Thr	V	V	V:996 T:4
325 B	Boundary	N	N	N:651 T:232 D:64 A:53
328 B	Boundary	L	M	M:556 L:407 K:37
332 B	Glu	I	E	E:1000

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 273 B, 275 B, 302 B, 323 B, 131 C

Table 25

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
240 B	All 20	V	T	A	T	A	A	A	A	T	A	A
263 B	All 20	V	T	W	T	T	A	T	T	T	L	L
266 B	All 20	V	L	A	T	T	V	L	T	T	L	V
325 B	All 20	N	A	N	A	A	N	A	A	A	A	A
328 B	All 20	L	L	K	L	L	L	L	L	L	L	L

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332 A	Glu	I	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1E4K template structure; + carbohydrate; floated 273 B, 275 B, 302 B, 323 B, 131 C

Table 26

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
240 A	Core + Thr	V	V	V:876 T:109 M:15
263 A	Core + Thr	V	V	V:913 T:87
266 A	Core + Thr	V	V	V:969 T:31
325 A	Boundary	N	V	V:491 N:236 T:187 A:35 D:32 S:19
328 A	Boundary	L	L	L:321 W:290 M:271 F:49 K:46 R:23
332 A	Glu	I	E	E:1000

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 273 A, 275 A, 302 A, 323 A, 158 C

Table 27

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
240 A	All 20	V	A	T	A	A	T	T	A	A	A	T
263 A	All 20	V	T	T	V	V	T	V	L	L	V	T
266 A	All 20	V	V	V	V	V	V	V	V	V	V	V
325 A	All 20	N	Q	N	Q	Q	Q	Q	Q	Q	N	N
328 A	All 20	L	K	M	K	K	K	K	K	K	K	K
332 A	Glu	I	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1E4K template structure; + carbohydrate; floated 273 A, 275 A, 302 A, 323 A, 158 C

[196] Computational screening calculations were aimed at designing Fc variants to optimize the conformation of the N297 carbohydrate and the Cγ2 domain. By exploring energetically favorable substitutions at positions that interact with carbohydrate, variants can be engineered that sample new, potentially favorable carbohydrate conformations. Fc residues F241, F243, V262, and V264 mediate the Fc/carbohydrate interaction and thus are target positions. The results of these design calculations are presented in Table 28.

Table 28

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
241 A	Core	F	Y	Y:172 M:162 L:144 F:140 W:110 I:97 A:91 V:84
243 A	Core	F	Y	Y:211 L:204 W:199 F:160 M:141 A:85
262 A	Core	V	M	M:302 I:253 V:243 A:202
264 A	Core	V	F	I:159 M:152 V:142 L:140 W:136 F:120 Y:104 A:47

PDA® technology, 1IIS template structure; - carbohydrate

[197] Computational screening calculations were aimed at designing Fc variants to optimize the angle between the Cγ3 and Cγ2 domains. Residues P244, P245, P247, and W313, which reside at the Cγ2/Cγ3 interface, appear to play a key role in determining the Cγ2-Cγ3 angle and the flexibility of the domains relative to one another. By exploring energetically favorable substitutions at these positions, variants can be designed that sample new, potentially favorable angles and levels of flexibility. The results of these design calculations are presented in Table 29.

Table 29

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
244 A	Boundary	P	H	K:164 H:152 R:110 M:100 S:92 N:57 A:54 D:50 Q:49 T:46 E:37 V:30 L:27 W:23 F:9
245 A	Boundary	P	A	A:491 S:378 N:131
247 A	Boundary	P	V	V:156 T:125 K:101 E:87 Q:79 R:78 S:76 A:72 D:72 H:60 M:47 N:47
313 A	Boundary	W	W	W:359 F:255 Y:128 M:114 H:48 K:29 T:24 A:11 E:10 V:10 S:9 Q:3

PDA® technology; 1IIS template structure; - carbohydrate

[198] In addition to the above calculations using PDA® and SPA™ computational screening methods, additional calculations using solely an electrostatic potential were used to computationally screen Fc variants. Calculations with Coulomb's law and Debye-Huckel scaling highlighted a number of positions in the Fc for which amino acid substitutions would favorably affect binding to one or more FcγRs, including positions for which replacement of a neutral amino acid with a negatively charged amino acid may enhance binding to FcγRIIIa, and for which replacement of a positively charged amino acid with a neutral or negatively charged amino acid may enhance binding to FcγRIIIa. These results are presented in Table 30.

Table 30

Replacement of a + residue with a - residue	Replacement of a neutral residue with a - residue
H268	S239
K326	Y296
K334	A327
	I332

Coulomb's law and Debye-Huckel scaling; 1IIS template structure; + carbohydrate

[199] Computational screening calculations were carried out to optimize aglycosylated Fc, that is to optimize Fc structure, stability, solubility, and Fc/FcγR affinity in the absence of the N297 carbohydrate. Design calculations were aimed at designing favorable substitutions in the context of

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the aglycosylated Fc template structure at residue 297, residues proximal to it, residues at the Fc/FcγR interface, and residues at the Fc/carbohydrate interface. Variable positions were grouped in different sets of interacting variable positions and designed in separate sets of calculations, and various template structures were used. For many of the variable position sets, calculations were carried out using both the PDA® and SPA™ computational screening methods. The results of these calculations and relevant information are presented in Tables 31 - 53 below.

Table 31

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
265 A	Boundary XM	D	Y	Y:531 F:226 W:105 H:92 K:21 D:16 E:6 T:3
297 A	Boundary XM	N	D	A:235 S:229 D:166 E:114 N:92 Y:57 F:55 Q:25 H:10 T:7 K:6 L:3 R:1
299 A	Boundary XM	T	L	L:482 Y:186 F:131 T:55 S:51 K:31 H:22 A:18 E:14 Q:10
297 B	Boundary XM	N	I	I:299 K:147 V:85 R:82 W:71 N:65 D:35 E:35 Q:34 S:32 L:31 H:30 T:28 A:26

PDA® technology; 1IIS template structure; - carbohydrate; floated 122 C, 129 C, 132 C, 155 C

Table 32

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
297 A	All 20	N	A	T	A	E	K	K	A	A	N	N
299 A	All 20	T	S	K	S	K	F	F	F	F	F	S
297 B	All 20	N	K	K	K	K	K	K	K	K	K	K

SPA™ technology; 1IIS template structure; - carbohydrate; floated 122 C, 129 C, 132 C, 155 C

Table 33

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Surface	S	E	E:928 Q:65 D:7
265 A	Boundary XM	D	W	W:709 Y:248 F:43
296 A	Surface	Y	H	H:449 Y:146 E:137 D:89 K:64 N:32 T:30 R:25 Q:23 S:5
297 A	Surface	N	E	E:471 H:189 D:102 T:97 K:96 R:22 Q:15 S:8
298 A	Boundary XM	S	R	R:353 T:275 K:269 A:56 S:38 E:5 Q:2 H:2
299 A	Boundary XM	T	F	Y:398 F:366 L:217 H:15 K:4

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 128 C, 132 C, 155 C

Table 34

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	All 20	S	E	Q	Q	E	Q	Q	Q	Q	Q	Q
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
296 A	All 20	Y	D	Q	N	N	Q	N	N	N	Q	N

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297 A	All 20	N	A	A	N	A	D	D	E	N	N	E
298 A	All 20	S	K	K	K	S	K	K	K	K	S	K
299 A	All 20	T	S	Y	F	S	Y	F	K	F	S	K

SPA™ technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 128 C, 132 C, 155 C

Table 35

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Surface	S	E	E:417 T:122 D:117 Q:94 R:84 S:63 K:47 H:29 N:19 A:8
265 B	Boundary XM	D	W	W:865 Y:79 F:55 K:1
296 B	Surface	Y	Y	Y:549 H:97 D:80 S:75 N:48 E:45 K:32 R:30 Q:28 A:16
297 B	Surface	N	R	R:265 H:224 E:157 K:154 Q:75 D:47 T:34 N:24 S:13 A:7
298 B	Boundary XM	S	V	V:966 D:10 T:8 A:8 N:4 S:4
299 B	Boundary XM	T	Y	Y:667 F:330 H:3

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 125 C, 129 C, 152 C

Table 36

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	S	R	E	K	S	S	E	E	E	K
265 B	All 20	D	A	D	K	Y	A	A	F	F	K	Y
296 B	All 20	Y	A	A	A	A	A	A	A	A	A	A
297 B	All 20	N	T	S	T	T	E	E	E	S	E	E
298 B	All 20	S	G	G	G	G	G	G	G	G	G	G
299 B	All 20	T	L	F	E	E	Y	F	Y	F	Y	Y

SPA™ technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 125 C, 129 C, 152 C

Table 37

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Surface	S	E	E:868 Q:92 D:38 K:1 N:1
265 A	Boundary XM	D	W	W:575 Y:343 F:66 H:15 K:1
296 A	Surface	Y	H	H:489 Y:103 R:98 K:97 Q:64 D:63 T:41 N:38 E:7
297 A	Asp	N	D	D:1000
298 A	Boundary XM	S	R	R:340 K:262 T:255 A:59 S:57 E:11 Q:10 H:6
299 A	Boundary XM	T	F	Y:375 F:323 L:260 H:24 K:18

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 128 C, 132 C, 155 C

Table 38

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	All 20	S	E	Q	E	E	E	E	E	E	Q	E

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265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
296 A	All 20	Y	E	N	Q	E	N	Q	Q	Q	Q	N
297 A	Asp	N	D	D	D	D	D	D	D	D	D	D
298 A	All 20	S	K	S	K	S	K	K	K	S	K	K
299 A	All 20	T	S	K	Y	S	F	F	F	F	F	K

SPA™ technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 128 C, 132 C, 155 C

Table 39

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Surface	S	E	E:318 Q:123 T:109 D:108 R:93 S:89 K:69 N:40 H:38 A:13
265 B	Boundary XM	D	W	W:745 Y:158 F:85 K:9 E:1 R:1 H:1
296 B	Surface	Y	Y	Y:390 H:127 S:83 R:81 K:78 N:65 D:55 E:49 Q:44 A:26 T:2
297 B	Asp	N	D	D:1000
298 B	Boundary XM	S	V	V:890 T:35 A:29 D:19 S:16 N:10 E:1
299 B	Boundary XM	T	Y	Y:627 F:363 H:10

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 125 C, 129 C, 152 C

Table 40

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	K	E	E	Q	E	K	Q	E	K	Q
265 B	All 20	D	F	K	K	A	K	Y	W	K	L	F
296 B	All 20	Y	A	A	A	A	A	A	A	A	A	A
297 B	Asp	N	D	D	D	D	D	D	D	D	D	D
298 B	All 20	S	G	G	G	G	G	G	G	G	G	G
299 B	All 20	T	Y	Y	Y	Y	Y	Y	F	F	Y	Y

SPA™ technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 125 C, 129 C, 152 C

Table 41

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Boundary XM	S	E	E:312 L:148 D:102 Q:98 K:64 I:61 S:57 A:44 T:39 N:29 R:23 V:18 W:5
265 A	Boundary XM	D	W	W:363 Y:352 F:139 H:77 K:39 R:14 D:11 E:4 Q:1
297 A	Asp	N	D	D:1000
299 A	Boundary XM	T	Y	Y:309 F:224 L:212 H:96 K:92 E:28 Q:20 R:16 T:2 S:1

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 132 C, 155 C

Table 42

Position	Considered	WT	1	2	3	4	5	6	7	8	9	10

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	Amino Acids											
239 A	All 20	S	E	L	L	L	E	E	E	Q	L	E
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
297 B	Asp	N	D	D	D	D	D	D	D	D	D	D
299 A	All 20	T	S	K	K	F	F	F	K	F	K	F

SPA™ technology; D129G 1IIS template structure; - carbohydrate; floated 120 C, 122 C, 132 C, 155 C

Table 43

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Boundary XM	S	L	L:194 T:122 S:120 E:111 D:79 K:71 A:62 Q:57 R:43 H:43 N:37 I:24 W:24 V:13
265 B	Boundary XM	D	W	Y:248 W:233 F:198 K:84 D:57 E:55 H:42 R:28 Q:20 A:10 T:10 N:8 S:7
297 B	Asp	N	D	D:1000
299 B	Boundary XM	T	Y	Y:493 F:380 H:76 T:31 E:10 D:4 A:3 S:3

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 129 C, 152 C

Table 44

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	R	E	P	L	L	F	P	P	L	L
265 B	All 20	D	D	K	S	F	S	Y	A	M	A	D
297 B	Asp	N	D	D	D	D	D	D	D	D	D	D
299 B	All 20	T	Y	Y	Y	Y	E	Y	Y	Y	Y	Y

SPA™ technology; D129G 1E4K template structure; - carbohydrate; floated 117 C, 119 C, 129 C, 152 C

Table 45

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 A	Boundary XM	S	E	E:251 L:125 D:120 Q:112 S:73 K:65 I:61 A:58 T:45 N:35 R:28 V:23 W:4
265 A	Boundary XM	D	Y	Y:216 H:153 K:135 D:109 W:104 F:86 R:54 T:38 E:29 Q:22 A:21 N:17 S:13 L:3
297 A	Asp	N	D	D:1000

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 299 A, 120 C, 122 C, 132 C, 155 C

Table 46

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 A	All 20	S	S	L	E	L	Q	Q	E	Q	Q	E
265 A	All 20	D	G	G	G	G	G	G	G	G	G	G
297 A	Asp	N	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1IIS template structure; - carbohydrate; floated 299 A, 120 C, 122 C, 132 C, 155 C

Table 47

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
239 B	Boundary XM	S	L	L:158 S:137 T:125 E:115 D:86 K:75 A:62 Q:56 H:43 R:39 N:35 W:30 I:24 V:15
265 B	Boundary XM	D	Y	Y:188 W:159 F:156 D:122 K:77 E:71 H:61 Q:44 R:39 A:24 S:22 N:19 T:18
297 B	Asp	N	D	D:1000

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 299 B, 117 C, 119 C, 129 C, 152 C

Table 48

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
239 B	All 20	S	S	E	P	P	E	S	P	L	F	L
265 B	All 20	D	A	K	A	M	K	F	Y	D	F	F
297 B	Asp	N	D	D	D	D	D	D	D	D	D	D

SPA™ technology; D129G 1E4K template structure; - carbohydrate; floated 299 B, 117 C, 119 C, 129 C, 152 C

Table 49

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
297 A	Asp	N	D	D:1000
299 A	Boundary XM	T	Y	T:123 Y:64 H:64 K:64 Q:64 F:64 R:63 D:63 E:63 S:63 L:63 N:62 I:57 A:54 V:52 W:17

PDA® technology; D129G 1IIS template structure; - carbohydrate; floated 239 A, 265 A, 120 C, 122 C, 132 C, 155 C

Table 50

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
297 A	Asp	N	D	D	D	D	D	D	D	D	D	D
299 A	All 20	T	K	K	K	K	F	F	K	K	K	K

SPA™ technology; D129G 1IIS template structure; - carbohydrate; floated 239 A, 265 A, 120 C, 122 C, 132 C, 155 C

Table 51

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
297 B	Asp	N	D	D:1000
299 B	Boundary XM	T	Y	T:123 F:64 Y:64 H:64 S:63 N:61 Q:61 D:61 E:60 K:58 V:57 A:57 R:54 I:52 L:51 W:50

PDA® technology; D129G 1E4K template structure; - carbohydrate; floated 239 B, 265 B, 117 C, 119 C, 129 C, 152 C

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Table 52

Position	Considered Amino Acids	WT	1	2	3	4	5	6	7	8	9	10
297 B	Asp	N	D	D	D	D	D	D	D	D	D	D
299 B	All 20	T	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

SPA™ technology; D129G 1E4K template structure; - carbohydrate; floated 239 B, 265 B, 117 C, 119 C, 129 C, 152 C

[200] Computational screening calculations were carried out to optimize aglycosylated Fc by designing favorable substitutions at residues that are exposed to solvent in the absence of glycosylation such that they are stable, maintain Fc structure, and have no tendency to aggregate. The N297 carbohydrate covers up the exposed hydrophobic patch that would normally be the interface for a protein-protein interaction with another Ig domain, maintaining the stability and structural integrity of Fc and keeping the Cγ2 domains from aggregating across the central axis. Key residues for design are F241, F243, V262, and V264, which reside behind the carbohydrate on Cγ2, in addition to residues such as L328, I332, and I336, which are exposed nonpolar residues facing inward towards the opposed Cγ2 domain, that were considered in previously presented calculations. The importance of these Cγ2 residues is supported by noting that the corresponding residues in the Cγ3 domain by sequence alignment either mediate the nonpolar interaction between the two Cγ3 domains or are buried in the Cγ3 core. The results of these design calculations are presented in Table 53.

Table 53

Position	Considered Amino Acids	WT	Ground State	Sequences Around Ground State
241 A	Surface	F	E	E:190 R:172 K:138 H:117 T:93 Q:91 D:85 S:49 N:49 A:16
243 A	Surface	F	R	R:190 H:164 Q:152 E:149 K:92 T:71 D:64 N:58 S:42 A:18
262 A	Surface	V	D	D:416 E:164 N:138 Q:87 T:83 R:44 S:32 K:24 A:11 H:1
264 A	Surface	V	H	R:368 H:196 K:147 E:108 Q:68 T:34 N:33 D:25 S:15 A:6

PDA® technology; 1IIS template structure; - carbohydrate

[201] In a final set of calculations, a SPA™ computational screening method was applied to evaluate the replacement of all chosen variable positions with all 20 amino acids. The lowest energy rotamer conformation for all 20 amino acids was determined, and this energy was defined as the energy of substitution for that amino acid at that variable position. These calculations thus provided an energy of substitution for each of the 20 amino acids at each variable position. These data were

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useful for a variety of design goals aimed at both glycosylated and aglycosylated Fc, including optimization of Fc/FcγR affinity, Fc stability, Fc solubility, carbohydrate conformation, and hinge conformation. Furthermore, because these calculations provide energies for both favorable and unfavorable substitutions, they guide substitutions that may enable differential binding to activating versus inhibitory FcγRs. Various template structures were used, and calculations explored substitutions on both chains. The results of these calculations and relevant parameters and information are presented in Tables 54 - 59 below. Column 1 lists the variable positions on chain A and B of the 1IIS template structure. Column 2 lists the wild-type amino acid identity at each variable position. The remaining 20 columns provide the energy for each of the natural 20 amino acids (shown in the top row). All substitutions were normalized with respect to the lowest energy substitution, which was set to 0 energy. For example in Table 54, for L235 on chain A, serine is the lowest energy substitution, and L235A is 0.9 kcal/mol less stable than L235S. Extremely high energies were set to 20 kcal/mol for energies between 20 – 50 kcal/mol, and 50 kcal/mold for energies greater than 50 kcal/mol. Favorable substitutions may be considered to be the lowest energy substitution for each position, and substitutions that have small energy differences from the lowest energy substitution, for example substitutions within 1-2, 1-3, 1-5, or 1-10 kcal/mol.

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Table 54

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
235 A	L	0.9	2.8	2.8	1.5	3.2	3.2	3.4	4.9	1.6	2.1	3.2	0.9	0.3	1.3	0.7	0.0	1.7	4.3	6.5	3.2
236 A	G	0.0	1.9	5.1	6.7	10.0	2.3	4.3	17.2	5.7	20.0	4.6	3.2	12.6	5.6	6.1	0.6	6.2	12.0	6.7	20.0
237 A	G	20.0	20.0	20.0	50.0	50.0	0.0	50.0	50.0	20.0	50.0	20.0	20.0	50.0	50.0	50.0	20.0	20.0	50.0	50.0	50.0
239 A	S	0.2	4.3	2.6	0.0	12.8	4.5	6.9	11.3	1.7	0.1	2.1	1.7	7.9	1.2	2.6	0.3	5.7	11.0	20.0	20.0
265 A	D	9.0	8.1	6.3	7.8	5.1	0.0	7.3	50.0	8.2	9.9	7.7	6.0	50.0	9.0	8.5	7.8	20.0	50.0	20.0	5.8
267 A	S	2.1	3.3	7.3	1.4	50.0	7.3	20.0	20.0	0.9	2.2	5.0	4.8	0.0	2.2	3.1	2.9	20.0	20.0	50.0	50.0
269 A	E	0.5	2.1	1.3	0.6	1.6	3.9	2.0	1.2	1.1	1.3	2.7	0.0	50.0	0.6	1.1	0.3	0.8	1.0	5.6	1.2
270 A	D	0.3	2.8	2.3	2.0	4.0	4.0	3.4	2.4	1.2	0.0	2.3	2.1	20.0	2.0	2.3	1.4	1.8	4.2	5.4	6.0
296 A	Y	2.7	2.0	1.4	0.0	50.0	0.0	50.0	4.6	2.1	2.4	3.3	1.2	50.0	0.2	1.5	1.3	4.6	4.4	16.3	18.2
298 A	S	0.7	2.4	6.7	3.4	20.0	3.9	20.0	6.7	0.0	4.1	1.4	4.1	50.0	1.8	1.1	0.2	2.2	6.3	17.8	20.0
299 A	T	0.6	2.8	11.5	10.1	20.0	6.1	20.0	10.7	7.1	20.0	4.3	6.8	50.0	6.3	12.0	0.0	3.0	7.1	14.8	20.0
234 B	L	2.1	3.2	4.1	4.2	1.6	5.3	0.1	0.7	0.6	1.0	2.0	1.7	50.0	2.8	0.3	2.3	1.7	2.6	13.0	0.0
235 B	L	0.6	2.3	2.5	0.7	5.4	4.8	1.4	3.6	0.1	0.0	2.0	1.7	16.6	0.5	1.2	0.7	0.7	5.3	6.8	5.5
236 B	G	3.1	1.3	4.4	8.2	5.2	0.0	1.9	20.0	3.1	20.0	4.1	2.7	50.0	3.7	16.0	1.2	20.0	20.0	20.0	11.3
237 B	G	20.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
239 B	S	0.9	2.4	3.4	1.8	5.4	5.6	2.7	3.0	0.9	0.0	2.0	1.6	50.0	1.8	1.8	1.4	1.4	5.1	20.0	5.3
265 B	D	4.5	5.1	4.6	4.6	4.9	0.0	3.8	9.0	2.0	2.5	4.1	2.1	50.0	4.5	5.1	4.4	5.9	9.2	11.4	5.8
327 B	A	1.8	3.4	4.7	3.9	20.	7.0	20.	20.	0.8	0.0	1.9	1.5	20.	3.0	2.6	3.2	20.	20.	20.	20.

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328 B	L	3.7	3.6	4.0	3.7	50.0	8.4	6.8	50.0	3.8	0.0	2.1	4.1	50.0	3.6	8.1	4.9	3.0	12.5	50.0	50.0
329 B	P	3.4	8.6	20.0	20.0	50.0	8.0	16.8	50.0	20.0	20.0	16.9	20.0	0.0	20.0	20.0	1.3	17.1	16.5	50.0	50.0
330 B	A	0.5	2.0	2.6	0.5	2.4	3.8	1.4	4.2	0.0	2.0	2.2	0.8	20.0	0.1	0.6	0.9	0.3	5.1	8.0	2.7
332 B	I	1.5	2.7	1.2	1.6	11.9	6.8	12.9	1.2	2.9	0.0	1.4	1.7	50.0	1.3	4.9	1.8	1.7	3.0	20.0	20.0

SPA™ technology; 1IIS template structure; + carbohydrate atoms, no floated positions

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Table 55

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
235 A	L	0.9	2.8	2.6	1.7	3.3	3.3	3.4	5.0	1.6	2.1	3.3	1.0	0.3	1.4	1.8	0.0	1.9	3.6	6.6	3.3
236 A	G	0.0	1.7	5.2	6.0	11.3	2.3	4.4	17.2	5.8	19.0	4.9	3.3	8.2	5.6	6.0	0.8	5.6	11.8	6.6	20.0
237 A	G	20.0	20.0	20.0	50.0	50.0	0.0	50.0	50.0	20.0	50.0	20.0	20.0	50.0	50.0	50.0	20.0	20.0	50.0	50.0	50.0
238 A	P	8.6	8.0	10.5	13.4	6.4	0.0	5.0	50.0	12.4	11.3	9.7	9.3	3.2	12.4	20.0	8.6	50.0	50.0	20.0	8.4
239 A	S	0.1	4.2	2.5	0.0	20.0	4.5	9.0	10.8	1.8	0.2	2.1	1.8	9.1	1.3	2.5	0.3	5.7	10.7	20.0	19.7
240 A	V	1.3	2.4	2.3	6.3	20.0	7.2	20.0	5.1	10.8	6.2	5.7	2.0	1.1	9.5	13.1	2.5	0.5	0.0	20.0	20.0
241 A	F	0.1	1.6	1.2	0.3	0.2	4.1	1.2	10.0	1.3	0.1	2.1	0.4	14.7	0.5	1.1	0.1	0.0	8.3	3.6	0.4
242 A	L	3.0	3.4	5.5	8.3	14.4	8.5	11.1	3.3	13.9	2.2	2.7	5.5	0.9	7.9	17.1	3.8	2.3	0.0	20.0	17.5
243 A	F	1.6	2.2	2.7	0.2	1.4	5.6	2.5	0.0	2.2	2.0	3.0	2.3	10.2	0.5	1.6	1.3	0.9	1.2	5.3	1.6
244 A	P	1.2	1.8	3.8	0.8	10.2	3.8	4.6	20.0	0.2	2.9	2.0	2.8	2.0	0.9	1.7	0.0	19.3	20.0	7.6	12.2
245 A	P	3.9	20.0	20.0	20.0	20.0	9.1	20.0	20.0	20.0	20.0	20.0	20.0	0.0	20.0	20.0	8.0	20.0	50.0	20.0	20.0
246 A	K	1.3	2.7	2.0	2.0	2.9	5.7	2.9	1.4	1.4	1.5	3.1	0.2	0.0	1.2	1.5	1.7	1.4	1.2	5.4	3.0
247 A	P	1.2	2.1	0.3	0.7	4.0	3.9	3.7	1.8	1.6	1.7	3.3	0.0	0.5	0.9	1.5	0.7	1.1	1.3	6.9	3.7
248 A	K	0.9	2.7	1.5	0.8	3.1	4.7	3.4	3.3	2.0	1.9	2.6	1.2	3.6	1.5	2.3	0.7	0.0	2.5	5.6	2.7
249 A	D	1.2	3.7	1.6	0.0	20.0	7.3	19.7	50.0	1.7	20.0	2.2	1.4	20.0	1.5	3.4	2.5	18.3	50.0	20.0	20.0
250 A	T	0.0	1.8	3.8	5.8	50.0	6.0	20.0	4.5	6.3	6.3	0.3	3.2	50.0	8.7	9.3	1.8	1.3	1.9	20.0	50.0
251 A	L	1.1	1.9	1.2	0.5	5.8	5.1	1.9	5.6	0.9	0.7	2.4	1.4	50.0	0.0	1.4	0.5	0.8	6.9	8.9	5.8
252	M	0.3	1.2	0.6	0.0	3.0	3.8	3.4	3.9	1.0	0.3	2.2	0.3	17.	0.1	1.1	0.1	0.2	4.6	4.2	3.3

A														4						
253 A	I	0.7	1.7	1.1	0.2	1.8	3.5	2.2	2.0	0.3	1.2	0.8	0.8	0.3	0.0	1.1	0.3	0.5	2.8	2.4	1.9
254 A	S	0.7	1.7	0.4	0.7	2.2	3.6	2.0	0.3	1.2	1.9	2.4	0.0	20.0	0.3	1.2	0.3	0.8	0.7	3.8	1.9
255 A	R	1.4	2.8	2.4	2.5	0.2	5.4	1.1	17.0	1.0	2.2	1.5	1.7	50.0	2.1	0.0	2.3	50.0	17.2	4.0	0.5
256 A	T	0.6	1.8	1.2	1.1	2.7	3.4	2.1	1.4	0.7	1.5	2.4	0.0	0.4	0.1	0.2	0.4	0.9	1.2	5.6	2.7
257 A	P	0.0	7.8	20.0	12.9	50.0	6.2	50.0	20.0	12.3	12.8	14.4	20.0	0.1	13.1	20.0	2.9	16.0	20.0	50.0	50.0
258 A	E	0.0	1.6	4.8	2.6	1.0	4.3	2.2	14.8	4.4	6.2	3.2	2.9	10.4	7.4	6.0	1.0	6.2	17.6	20.0	1.0
259 A	V	3.9	4.3	5.1	8.7	20.0	10.3	6.8	2.3	9.6	2.8	6.2	4.1	50.0	9.2	20.0	5.2	2.1	0.0	20.0	20.0
260 A	T	1.7	2.3	3.3	1.1	20.0	6.6	8.6	0.0	0.2	1.8	2.8	1.8	1.1	0.8	0.9	1.7	0.4	1.9	7.1	20.0
261 A	C	0.0	20.0	20.0	20.0	20.0	3.9	20.0	20.0	20.0	20.0	20.0	20.0	50.0	20.0	20.0	3.6	20.0	20.0	20.0	20.0
262 A	V	1.9	3.2	0.0	3.3	20.0	7.2	20.0	8.3	2.9	2.9	2.2	0.6	50.0	3.8	5.2	3.4	3.0	1.7	20.0	20.0
263 A	V	2.2	2.7	6.0	17.4	20.0	8.8	20.0	10.0	7.1	7.6	16.9	5.2	50.0	19.8	17.7	2.8	1.4	0.0	20.0	20.0
264 A	V	1.9	3.3	2.8	2.2	0.0	6.4	2.1	0.7	2.6	0.9	2.7	2.1	2.3	2.6	2.7	2.2	1.1	0.6	3.9	0.1
265 A	D	9.0	8.1	5.9	8.6	5.3	0.0	7.3	50.0	7.9	9.7	7.5	5.5	50.0	10.2	8.6	7.9	20.0	50.0	20.0	5.7
266 A	V	4.9	5.3	7.1	12.1	20.0	11.2	20.0	0.4	12.2	20.0	8.8	7.1	50.0	12.2	20.0	6.1	3.8	0.0	20.0	20.0
267 A	S	2.3	3.5	7.2	1.3	50.0	7.4	20.0	20.0	0.7	1.4	3.9	4.7	0.0	2.3	3.1	3.0	20.0	20.0	50.0	50.0
268 A	H	1.2	1.9	2.2	1.5	3.7	5.0	4.9	0.4	0.5	3.7	2.7	1.7	0.0	1.4	1.7	1.1	0.2	0.9	6.1	3.7
269 A	E	0.3	1.9	1.3	0.5	1.3	3.7	1.9	1.1	0.8	1.2	2.5	0.0	50.0	0.6	0.8	0.2	0.6	0.7	4.0	1.0
270 A	D	0.2	2.6	2.1	1.9	5.2	3.9	3.1	2.1	1.2	0.0	2.2	1.9	20.0	1.9	1.8	1.2	1.7	4.1	5.1	7.0

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Table 55 (continued)

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
271 A	P	0.0	5.3	8.1	9.3	20.0	3.1	9.1	20.0	6.0	9.5	5.3	7.3	5.9	5.9	5.9	1.6	4.1	15.2	20.0	20.0
272 A	Q	0.8	1.9	0.9	1.2	3.0	3.2	3.7	3.7	1.6	1.8	3.2	0.3	50.0	1.1	1.6	0.0	1.0	3.5	4.0	3.4
273 A	V	1.2	2.9	1.8	20.0	20.0	7.1	20.0	6.8	20.0	20.0	20.0	0.0	2.8	20.0	20.0	2.1	1.4	1.7	20.0	20.0
274 A	K	0.4	1.8	1.4	0.8	1.9	3.9	2.4	1.4	0.7	1.1	2.9	0.9	20.0	0.0	0.1	0.0	0.4	0.7	3.3	2.3
275 A	F	8.0	9.5	10.3	9.5	0.0	13.5	5.1	10.1	6.2	6.3	6.0	9.1	6.1	9.1	15.1	9.6	7.2	6.1	13.5	4.3
276 A	N	1.3	2.4	2.4	2.2	0.8	5.1	0.8	1.2	0.6	2.3	2.5	1.8	50.0	1.6	2.5	1.2	0.0	0.3	4.2	3.6
277 A	W	5.5	7.4	8.4	6.4	15.4	11.2	3.2	8.2	1.9	3.9	3.6	6.6	3.5	5.5	15.4	6.9	6.1	14.1	0.0	20.0
278 A	Y	1.6	2.7	3.9	1.6	1.0	7.3	3.4	17.7	1.4	7.5	2.1	0.0	50.0	1.9	2.2	2.6	9.9	20.0	15.8	1.4
279 A	V	3.1	4.1	4.0	2.2	20.0	8.1	9.7	8.5	0.0	1.4	3.1	3.3	20.0	1.9	4.6	4.3	3.4	4.2	20.0	20.0
280 A	D	1.8	2.6	2.7	0.2	11.5	2.9	8.8	20.0	3.4	3.2	2.8	3.8	50.0	0.0	3.7	0.6	6.8	12.7	11.9	11.4
281 A	G	50.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
282 A	V	0.9	2.1	1.6	1.1	2.9	4.2	3.5	1.4	1.5	1.8	3.6	0.4	18.9	0.5	1.0	0.0	0.6	0.9	4.7	3.1
283 A	E	0.7	1.6	0.7	0.5	1.0	4.4	1.4	0.4	1.2	1.8	1.9	0.0	0.4	0.6	1.5	0.4	0.3	1.2	4.1	0.9
284 A	V	0.0	2.2	3.1	1.2	20.0	5.0	20.0	4.0	0.7	2.6	0.8	2.6	50.0	0.8	0.7	0.8	0.1	1.5	20.0	20.0
285 A	H	0.2	1.4	3.1	1.3	3.0	2.0	2.4	3.6	1.1	2.6	3.0	0.7	2.2	0.2	0.8	0.0	1.1	4.7	4.9	4.0
286 A	N	0.8	2.5	1.2	1.1	2.4	4.7	2.7	2.1	0.0	0.7	1.8	0.6	20.0	1.2	0.7	0.9	1.7	2.1	5.2	2.7
287 A	A	0.6	2.6	5.8	3.3	10.4	5.4	9.1	11.3	0.0	4.4	1.3	3.6	50.0	2.6	2.3	1.0	1.9	12.5	9.1	10.4
288	K	0.8	2.6	2.0	1.3	3.0	3.4	3.8	2.3	1.4	1.7	2.5	0.3	50.	0.5	1.3	0.0	0.4	2.0	4.5	3.6

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A														0							
289 A	T	0.3	1.9	4.7	1.1	3.1	3.6	2.9	10.5	0.4	2.7	1.6	2.1	8.2	1.2	2.0	0.0	0.4	12.0	3.9	3.2
290 A	K	1.7	2.2	0.5	0.6	3.0	1.3	3.0	3.7	1.7	2.1	3.2	0.0	50.0	0.7	2.0	0.3	1.3	3.3	5.6	3.3
291 A	P	1.6	3.1	1.8	0.5	1.9	5.5	1.8	0.1	0.5	1.5	1.2	1.2	0.7	0.0	2.9	2.2	0.9	1.3	2.6	0.9
292 A	R	1.1	2.2	3.1	0.8	5.9	4.4	8.0	5.0	0.0	1.6	2.1	1.1	8.4	0.2	0.4	1.0	1.3	4.7	8.3	5.7
293 A	E	2.2	6.5	9.0	17.9	16.3	0.0	13.2	50.0	12.8	10.3	10.3	7.2	5.5	15.1	14.5	3.5	20.0	50.0	14.5	17.1
294 A	E	1.5	2.1	2.1	0.7	8.1	2.8	3.3	2.0	2.6	1.8	2.8	1.0	50.0	1.3	1.3	0.5	0.0	3.4	11.2	10.2
295 A	Q	50.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
296 A	Y	2.8	2.3	1.1	0.4	50.0	0.0	50.0	4.6	2.2	2.3	3.1	0.9	50.0	0.2	1.8	1.3	4.7	4.8	18.2	20.0
297 A	N	0.0	6.5	8.4	5.3	20.0	3.4	20.0	13.8	2.7	20.0	4.8	9.3	50.0	4.4	4.4	1.5	1.6	15.5	20.0	20.0
298 A	S	0.8	2.4	5.7	2.2	20.0	3.7	20.0	6.2	0.9	9.2	1.8	3.3	50.0	1.7	2.1	0.0	2.2	7.3	15.6	20.0
299 A	T	1.9	3.4	6.0	3.1	1.0	7.1	2.9	3.1	0.0	2.7	2.6	3.6	50.0	2.2	2.5	1.1	2.2	5.4	3.6	1.4
300 A	Y	2.8	2.9	2.7	4.5	20.0	4.0	7.5	13.1	1.2	0.0	2.2	2.3	50.0	3.3	4.0	2.6	1.1	1.1	11.0	2.4
301 A	R	3.0	3.5	3.8	2.8	0.8	3.4	1.8	0.0	1.3	0.7	2.6	2.5	50.0	2.6	2.3	2.9	1.8	0.9	9.8	1.8
302 A	V	2.7	4.6	6.7	3.9	2.8	8.9	1.2	6.9	2.7	2.0	2.2	4.8	50.0	4.7	3.2	4.3	7.7	3.8	0.0	8.4
303 A	V	0.0	2.2	3.3	1.0	6.7	4.5	5.3	1.4	2.5	3.1	2.0	3.1	1.0	2.1	2.9	0.4	0.4	2.9	10.9	6.2
304 A	S	0.0	12.1	10.8	20.0	20.0	6.2	20.0	20.0	17.2	20.0	11.9	16.6	50.0	20.0	16.6	2.2	14.2	17.9	20.0	20.0
305 A	V	1.1	2.3	3.3	1.2	0.3	5.4	1.2	0.0	0.9	1.1	2.8	1.1	3.9	1.1	1.4	1.2	0.9	0.0	0.8	0.8

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Table 55 (continued)

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
306 A	L	4.3	6.2	7.1	5.9	2.8	10.4	3.4	13.7	3.0	0.0	3.5	6.0	50.0	5.9	9.9	6.2	5.3	11.4	9.6	10.3
307 A	T	1.4	3.2	3.8	2.2	6.5	5.5	4.2	0.5	0.3	4.2	3.0	2.2	0.0	1.9	1.3	1.4	0.9	1.2	6.2	6.5
308 A	V	1.8	5.5	6.5	8.0	50.0	7.9	20.0	4.5	20.0	5.5	19.4	7.6	50.0	7.7	15.5	0.0	0.7	5.9	50.0	50.0
309 A	L	1.1	2.7	0.7	0.7	1.3	4.6	2.7	0.7	1.7	1.0	2.8	0.0	1.6	0.7	1.3	1.0	0.6	0.5	5.0	2.1
310 A	H	2.0	2.6	0.9	4.1	50.0	5.6	0.2	6.8	4.0	7.1	4.0	0.0	0.2	4.9	10.0	2.0	2.5	6.4	50.0	50.0
311 A	Q	0.6	2.5	1.6	1.6	2.5	4.3	1.6	1.4	0.6	0.9	2.9	0.9	1.7	0.8	0.9	0.0	0.3	2.2	4.6	2.0
312 A	N	5.4	5.1	5.9	1.3	20.0	0.0	20.0	10.0	3.4	4.8	3.3	7.1	50.0	2.7	3.9	4.1	3.2	11.9	20.0	20.0
313 A	W	4.6	6.4	5.5	5.6	1.1	10.8	5.0	11.0	5.8	5.2	7.6	5.4	50.0	4.8	12.9	6.0	3.8	6.6	0.0	2.6
314 A	L	2.1	2.9	4.3	2.2	5.7	6.1	7.9	5.4	0.7	0.0	1.7	2.3	50.0	1.6	1.6	3.0	4.7	6.3	8.0	6.0
315 A	D	0.3	1.4	1.5	0.1	3.3	4.2	1.9	1.8	0.8	0.5	1.8	0.6	50.0	0.0	0.7	0.0	0.9	2.4	6.2	3.7
316 A	G	50.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
317 A	K	0.0	14.0	18.4	17.9	50.0	5.0	50.0	20.0	8.5	12.5	12.7	20.0	15.9	17.2	13.5	2.8	9.2	20.0	50.0	50.0
318 A	E	2.0	3.0	2.7	1.7	2.7	6.7	2.6	0.0	1.1	1.6	1.3	1.7	20.0	1.4	2.6	2.2	1.3	0.0	6.1	9.5
319 A	Y	2.9	4.4	3.9	3.4	0.0	8.8	1.8	20.0	0.5	5.2	0.7	3.2	50.0	3.1	5.6	3.4	3.6	20.0	20.0	0.2
320 A	K	2.3	3.1	3.0	2.7	20.0	7.8	20.0	9.4	0.0	0.6	2.7	1.3	50.0	2.4	1.9	3.3	3.3	7.2	20.0	20.0
321 A	C	0.0	3.2	20.0	18.8	20.0	6.9	20.0	20.0	20.0	20.0	10.4	20.0	50.0	19.6	20.0	1.5	8.7	18.3	20.0	20.0
322 A	K	2.0	2.5	3.5	2.8	2.7	6.4	2.1	0.2	0.1	1.2	2.7	2.7	50.0	2.1	0.0	2.3	1.6	0.9	14.5	2.8
323	V	1.5	2.8	7.3	11.	20.	8.1	20.	6.0	9.6	20.	4.9	8.5	50.	13.	20.	2.8	1.6	0.0	20.	20.

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A					9	0		0			0			0	6	0				0	0
324 A	S	2.0	2.1	0.6	0.0	1.9	4.9	3.9	1.5	2.8	0.7	1.9	0.9	50.0	0.8	2.9	2.7	1.9	2.1	3.8	2.5
325 A	N	2.8	3.9	8.4	3.0	20.0	8.3	20.0	0.0	7.7	20.0	6.2	1.6	13.4	0.5	20.0	3.1	0.1	1.3	20.0	20.0
326 A	K	1.0	2.7	3.0	1.6	3.7	4.1	3.1	3.2	1.7	2.4	3.7	1.2	0.0	0.6	1.4	1.0	1.9	2.6	5.6	3.6
327 A	A	0.9	2.8	5.8	3.1	20.0	6.3	16.7	14.7	2.8	20.0	2.5	5.3	20.0	1.3	4.1	0.0	5.2	13.7	20.0	20.0
328 A	L	6.0	6.3	7.0	4.1	50.0	8.6	20.0	50.0	5.7	0.0	7.1	6.0	50.0	3.7	8.2	6.6	50.0	50.0	20.0	50.0
329 A	P	1.0	2.5	0.9	0.6	4.0	3.4	3.3	1.7	1.9	2.5	3.6	0.0	0.3	0.7	1.5	0.1	1.1	1.1	6.2	3.6
330 A	A	0.9	2.0	1.3	0.7	3.4	3.8	3.0	2.0	1.4	2.0	3.4	0.0	20.0	0.6	1.2	0.2	0.6	1.9	7.0	3.4
331 A	P	50.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
332 A	I	1.9	3.7	4.6	1.7	5.0	7.0	1.9	3.8	1.8	0.0	2.5	3.9	20.0	0.8	2.4	2.3	2.6	4.4	20.0	5.9
333 A	E	0.0	3.1	3.2	0.8	4.1	4.4	4.2	16.9	3.6	2.8	2.8	2.5	1.6	1.3	3.2	1.3	1.4	7.7	4.0	4.8
334 A	K	1.7	2.9	2.5	0.0	1.0	6.1	3.3	1.0	1.5	0.5	3.5	1.5	4.4	0.1	2.7	2.2	0.9	1.3	4.9	1.8
335 A	T	0.5	3.2	4.5	2.7	4.2	4.9	4.1	20.0	2.1	3.1	3.0	1.2	0.0	2.3	2.8	1.4	1.4	7.3	5.1	4.5
336 A	I	1.2	1.6	5.0	1.5	20.0	6.1	16.8	0.7	3.4	7.8	2.5	3.2	20.0	2.8	1.4	0.7	0.6	0.0	20.0	20.0
337 A	S	4.8	4.8	7.5	11.5	10.1	0.0	5.5	50.0	9.9	7.0	7.9	5.0	50.0	11.4	12.7	4.5	2.3	50.0	19.3	10.6
338 A	K	1.0	2.7	2.3	2.2	4.6	5.9	2.4	50.0	0.0	2.1	1.9	1.0	50.0	1.5	0.9	0.7	10.3	50.0	5.4	4.9
339 A	A	1.0	2.5	0.8	1.1	4.4	3.7	3.7	2.1	1.8	2.6	3.6	0.0	0.8	0.6	1.6	0.6	0.9	2.4	6.8	3.8
340 A	K	1.3	2.4	2.3	2.0	1.7	4.1	2.3	1.9	0.0	2.3	1.8	1.0	1.9	0.9	1.3	0.5	0.8	1.7	4.9	2.4

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Table 55 (continued)

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
232 B	P	1.3	3.2	2.2	2.2	4.1	2.9	3.6	1.8	2.1	2.8	3.9	1.1	0.0	1.1	1.6	0.7	1.4	3.0	6.2	4.1
233 B	E	0.5	2.2	1.7	0.5	2.6	3.7	2.9	4.4	1.4	1.1	3.2	0.6	2.7	0.4	1.6	0.0	1.2	6.9	5.5	2.6
234 B	L	2.9	4.0	4.8	4.9	2.0	6.1	0.8	1.5	0.0	1.9	2.7	2.6	20.0	3.6	1.2	3.1	2.5	3.4	13.4	0.5
235 B	L	0.6	2.3	2.4	0.9	5.7	4.9	1.4	3.7	0.0	0.0	1.9	1.9	17.3	0.6	1.4	0.8	0.7	5.2	7.8	5.3
236 B	G	3.6	2.5	5.1	11.8	6.8	0.0	2.8	20.0	5.0	20.0	4.5	3.5	50.0	5.5	19.9	2.6	20.0	20.0	20.0	14.1
237 B	G	20.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
238 B	P	3.5	4.7	8.5	4.2	20.0	9.8	20.0	0.0	5.6	9.6	4.6	8.1	1.3	5.8	20.0	4.9	4.4	1.3	20.0	20.0
239 B	S	1.0	2.5	3.4	2.0	7.2	5.7	3.1	3.1	0.6	0.0	2.0	1.9	50.0	1.7	1.1	1.5	1.5	5.2	20.0	5.2
240 B	V	0.1	2.3	7.0	11.9	20.0	6.5	20.0	8.1	12.7	20.0	12.0	7.6	0.0	11.6	20.0	1.2	1.9	0.8	20.0	20.0
241 B	F	0.0	2.0	1.4	0.8	1.0	4.0	2.0	6.5	1.1	0.6	2.3	0.2	50.0	0.3	1.5	0.1	0.9	5.7	4.1	1.1
242 B	L	2.2	3.3	6.5	6.6	6.9	7.9	4.3	0.0	8.7	3.9	4.8	5.3	0.0	9.1	6.8	2.9	1.1	0.5	20.0	8.7
243 B	F	0.8	2.6	1.9	1.7	0.8	4.9	2.0	3.6	1.2	0.8	2.5	0.0	50.0	1.6	2.7	0.1	1.8	3.9	4.3	1.0
244 B	P	1.1	2.1	4.0	1.1	11.9	3.5	5.4	20.0	1.4	3.2	3.0	2.0	1.8	1.2	1.3	0.0	19.6	20.0	9.1	11.0
245 B	P	3.2	20.0	20.0	20.0	20.0	8.6	20.0	20.0	20.0	20.0	20.0	20.0	0.0	20.0	20.0	6.0	20.0	50.0	20.0	20.0
246 B	K	0.5	2.6	1.4	1.2	2.1	4.4	1.6	0.6	0.9	1.4	2.5	0.2	0.3	0.2	0.3	0.1	0.0	2.0	4.9	2.4
247 B	P	0.8	2.5	0.7	1.0	3.6	3.9	2.6	6.2	1.8	2.1	2.9	0.3	0.0	0.8	1.5	0.3	0.7	9.5	6.6	3.4
248 B	K	0.2	2.2	0.2	0.6	2.2	4.1	2.5	2.4	1.7	1.0	2.2	0.0	1.3	0.8	1.7	0.5	0.7	2.8	4.7	2.3
249	D	2.8	3.3	0.0	4.6	10.	8.2	6.5	50.	4.6	6.2	4.4	0.5	50.	4.7	6.3	3.5	6.1	50.	20.	7.2

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B						1			0					0					0	0	
250 B	T	0.0	2.2	4.9	2.8	20.0	6.3	20.0	2.2	4.3	3.2	3.0	9.2	50.0	3.4	4.9	1.3	2.3	3.1	20.0	20.0
251 B	L	0.0	2.4	1.6	1.2	5.6	3.6	2.2	7.4	1.2	0.6	2.3	0.5	50.0	0.6	1.8	0.4	2.5	8.7	8.2	5.9
252 B	M	1.3	2.4	0.8	0.0	1.8	5.7	2.3	0.6	1.6	0.6	2.5	1.0	50.0	1.0	1.6	1.5	1.3	0.8	5.1	1.6
253 B	I	1.6	3.0	2.0	1.2	3.7	4.5	3.5	2.9	0.8	2.4	2.4	1.1	1.0	0.0	1.5	1.2	1.4	3.4	4.4	3.6
254 B	S	1.0	1.5	0.8	0.6	3.8	3.8	3.2	0.5	1.9	2.5	3.1	0.3	6.2	0.5	1.7	0.0	0.1	1.1	5.5	3.7
255 B	R	0.9	2.0	2.0	1.7	0.0	5.4	1.4	20.0	1.0	1.6	1.3	0.8	50.0	1.4	1.1	1.5	20.0	20.0	3.7	0.8
256 B	T	0.6	2.0	1.8	1.1	2.5	3.7	1.9	1.6	1.0	1.4	2.2	0.0	1.2	0.5	0.1	0.8	1.2	1.2	5.5	2.4
257 B	P	2.5	20.0	20.0	20.0	50.0	9.0	50.0	20.0	20.0	20.0	20.0	20.0	0.0	20.0	20.0	4.8	20.0	20.0	50.0	50.0
258 B	E	1.5	2.4	2.7	1.4	2.7	6.4	4.2	0.0	0.2	5.4	2.4	1.1	50.0	1.3	2.5	2.2	1.1	1.0	19.1	3.0
259 B	V	2.9	4.2	6.3	5.2	20.0	9.3	20.0	0.0	8.1	8.9	5.5	5.6	50.0	6.2	20.0	4.5	2.5	0.0	20.0	20.0
260 B	T	0.0	1.6	5.3	1.9	20.0	4.9	20.0	0.6	1.1	2.8	1.4	3.9	0.2	2.3	2.6	0.4	0.1	2.7	20.0	20.0
261 B	C	0.0	10.0	20.0	20.0	20.0	2.6	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	2.0	16.6	20.0	20.0	20.0
262 B	V	2.1	2.4	2.7	2.4	8.1	7.2	3.8	1.8	3.5	8.6	3.4	2.7	50.0	3.2	4.8	2.9	1.9	0.0	14.7	9.1
263 B	V	2.2	3.7	4.7	11.2	20.0	9.1	20.0	15.0	13.7	2.8	20.0	5.4	50.0	13.0	20.0	3.6	2.1	0.0	20.0	20.0
264 B	V	2.1	3.0	4.6	2.7	8.6	6.8	6.6	0.0	1.8	1.8	3.7	3.6	10.1	3.0	2.2	2.6	2.2	1.0	12.7	20.0
265 B	D	4.5	5.2	4.8	4.7	5.0	0.0	3.8	8.5	1.8	2.6	4.1	1.8	50.0	4.5	5.3	4.5	6.0	9.2	12.2	5.6
266 B	V	5.3	5.5	7.2	12.7	20.0	12.0	20.0	2.1	20.0	20.0	20.0	5.7	50.0	18.3	20.0	5.9	4.7	0.0	50.0	50.0
267 B	S	2.8	4.3	6.2	3.8	0.0	7.4	1.0	50.0	1.0	0.3	3.2	3.2	0.5	1.5	0.8	3.3	11.6	50.0	6.3	50.0

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Table 55 (continued)

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
268 B	H	2.6	3.7	5.1	4.1	4.9	6.0	1.8	2.6	0.0	2.5	3.8	2.6	3.4	2.1	1.8	2.5	3.8	2.7	7.8	5.5
269 B	E	0.4	2.4	1.7	0.8	2.8	3.7	2.6	1.0	1.0	1.6	3.0	0.0	12.8	0.5	0.7	0.3	0.7	0.6	5.1	2.7
270 B	D	0.0	1.6	1.1	7.3	4.8	4.3	2.6	20.0	3.8	14.5	3.8	1.2	5.9	6.3	2.1	0.3	1.9	5.4	16.3	5.6
271 B	P	1.1	3.3	5.6	3.4	4.1	5.5	4.2	20.0	1.9	3.6	3.9	3.3	7.4	2.7	0.0	1.5	2.2	5.2	4.8	4.4
272 B	Q	0.9	1.9	1.0	0.6	3.0	3.9	2.9	1.5	1.7	2.2	3.5	0.6	4.9	0.0	1.4	0.2	0.6	1.4	3.9	3.2
273 B	V	3.5	4.8	6.2	8.3	20.0	9.2	20.0	4.6	8.4	3.1	3.5	7.4	50.0	10.6	20.0	2.0	0.0	4.8	20.0	20.0
274 B	K	0.1	1.6	0.4	0.9	1.7	3.8	1.8	1.9	0.4	0.5	2.4	0.3	15.6	0.1	0.0	0.0	0.2	1.6	2.2	1.9
275 B	F	5.7	7.0	8.4	9.2	0.0	11.2	3.5	9.2	7.9	5.7	5.1	7.0	4.1	9.7	12.3	6.9	4.5	3.3	10.3	5.0
276 B	N	0.0	6.2	6.9	6.4	20.0	4.7	12.1	20.0	9.3	10.0	7.4	3.8	50.0	6.4	9.2	2.8	20.0	20.0	20.0	20.0
277 B	W	8.3	10.0	10.6	9.2	2.6	14.2	7.4	12.7	6.7	7.4	6.4	10.8	6.8	9.3	11.9	9.7	8.0	14.4	0.0	15.9
278 B	Y	0.0	2.3	17.4	4.0	50.0	5.1	50.0	20.0	2.8	20.0	2.1	12.6	11.0	4.4	2.0	0.8	2.5	19.8	20.0	4.2
279 B	V	3.1	3.5	4.2	2.9	20.0	8.5	13.9	0.4	0.0	2.9	2.0	3.4	20.0	1.4	4.0	4.2	2.4	1.2	20.0	20.0
280 B	D	0.5	3.0	2.1	1.5	6.7	3.1	4.7	12.6	2.9	1.6	2.9	1.6	20.0	1.4	3.1	0.0	2.7	5.5	8.1	7.3
281 B	G	5.6	5.8	5.5	4.8	7.9	0.0	7.2	6.5	5.3	5.7	7.1	3.4	50.0	4.0	5.3	3.6	3.2	6.4	10.3	7.6
282 B	V	0.4	1.9	1.1	0.6	2.9	4.1	2.1	1.3	1.0	1.4	2.9	0.2	50.0	0.0	0.7	0.0	0.4	0.7	6.1	2.8
283 B	E	0.6	1.9	4.3	1.7	6.7	4.2	5.2	2.9	0.5	4.4	0.3	2.5	0.0	1.5	1.6	1.0	1.5	3.9	7.9	6.7
284 B	V	0.4	2.4	2.5	1.1	20.0	5.9	20.0	1.1	1.2	6.2	0.8	2.4	50.0	1.5	3.3	0.0	1.5	1.8	20.0	20.0
285	H	1.3	2.4	2.1	1.7	2.4	3.4	1.2	1.8	0.7	2.3	2.7	0.0	1.6	0.9	0.8	0.5	0.4	2.0	5.8	2.4

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B																				
286 B	N	1.2	2.7	1.0	1.1	3.0	3.1	2.6	0.8	2.0	1.9	2.9	0.0	50.0	0.4	1.6	1.1	0.5	2.9	4.9	3.0
287 B	A	2.5	4.4	6.1	7.5	0.0	8.2	3.0	10.2	5.1	16.5	4.5	0.3	12.3	8.1	9.1	4.1	3.3	7.1	3.4	0.8
288 B	K	0.4	1.9	1.9	0.0	2.9	3.5	2.9	2.5	1.8	2.1	2.5	0.9	15.4	0.6	1.1	0.2	0.9	3.8	5.9	2.7
289 B	T	0.1	1.5	3.7	1.4	2.7	3.9	2.6	1.8	0.0	2.2	1.6	1.5	1.8	1.1	1.7	0.0	0.4	2.3	3.4	2.5
290 B	K	0.9	1.8	0.8	0.5	2.4	0.8	2.7	3.0	1.3	1.3	2.6	0.2	50.0	0.7	1.5	0.0	0.6	2.9	5.0	2.7
291 B	P	1.2	2.1	2.5	0.5	3.9	4.6	3.4	0.7	0.0	3.4	1.5	1.1	0.6	0.1	1.1	1.1	0.9	1.5	3.2	2.6
292 B	R	0.8	2.6	3.3	1.2	4.9	3.6	6.8	3.1	2.0	2.4	2.2	2.2	16.6	1.5	1.8	0.1	0.0	3.2	7.6	5.2
293 B	E	0.0	3.0	4.1	2.8	7.3	3.6	5.8	5.8	2.6	4.5	3.2	2.2	1.3	2.2	2.5	0.0	1.2	7.8	7.0	6.9
294 B	E	2.5	3.3	3.9	2.3	8.3	6.8	4.4	5.6	3.6	2.3	3.7	4.1	0.0	3.3	5.0	2.1	2.9	5.0	6.7	11.9
295 B	Q	1.1	2.2	1.9	0.6	3.8	2.8	3.1	8.0	1.4	2.2	3.4	1.0	0.4	0.4	1.1	0.0	3.9	6.6	6.1	3.5
296 B	Y	1.5	2.7	1.2	1.2	4.1	4.1	3.5	1.1	1.8	2.7	3.5	0.0	20.0	0.6	1.9	1.2	1.4	1.3	6.4	4.0
297 B	N	3.9	4.5	10.1	6.0	15.5	7.3	16.7	6.6	0.0	5.1	4.6	7.3	20.0	4.4	4.2	3.6	4.1	7.9	18.0	15.0
298 B	S	1.7	2.5	3.5	2.5	2.5	3.7	2.4	3.0	0.0	1.8	2.3	0.4	50.0	1.2	1.0	0.9	2.2	3.3	5.5	2.0
299 B	T	0.0	2.7	7.2	11.1	20.0	4.8	20.0	7.5	6.9	20.0	7.1	4.8	50.0	9.8	17.9	0.3	1.3	5.8	20.0	20.0
300 B	Y	3.8	5.2	8.0	4.3	20.0	8.6	20.0	12.2	0.0	4.3	3.2	6.5	50.0	4.0	3.8	4.3	3.6	9.1	20.0	6.4
301 B	R	1.2	1.8	2.3	1.1	20.0	5.8	11.3	5.2	0.3	5.0	2.0	1.6	14.1	0.6	0.4	1.8	1.1	0.0	17.9	20.0
302 B	V	3.5	4.8	5.5	3.7	0.2	9.6	1.1	0.5	2.6	3.5	2.5	4.7	9.6	4.1	0.6	4.3	2.0	0.0	20.0	0.2
303 B	V	0.2	0.0	0.1	1.0	20.0	5.0	13.3	5.1	1.7	10.4	1.9	2.0	8.6	2.0	4.7	0.6	0.5	1.3	20.0	20.0
304	S	1.5	2.3	8.2	20.	20.	7.6	20.	7.6	20.	20.	20.	6.3	50.	20.	20.	0.0	2.7	3.8	20.	20.

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Table 55 (continued)

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
304 B	S	1.5	2.3	8.2	20.0	20.0	7.6	20.0	7.6	20.0	20.0	20.0	6.3	50.0	20.0	20.0	0.0	2.7	3.8	20.0	20.0
305 B	V	0.1	1.2	3.3	1.1	20.0	4.6	20.0	3.2	1.1	11.0	1.5	1.8	50.0	0.6	2.0	0.6	0.0	0.7	20.0	20.0
306 B	L	4.7	6.8	6.3	4.3	10.4	11.1	7.8	4.2	3.0	0.0	3.8	5.7	13.4	4.4	14.1	5.5	4.3	6.0	20.0	12.1
307 B	T	1.5	3.0	2.7	1.7	4.1	5.2	3.0	1.6	1.9	3.1	3.4	1.7	0.0	1.7	1.9	1.5	1.4	2.0	4.4	4.3
308 B	V	0.0	0.6	7.6	20.0	20.0	6.6	20.0	20.0	16.1	15.1	20.0	12.4	50.0	20.0	20.0	1.2	3.6	4.3	20.0	20.0
309 B	L	1.4	3.0	2.2	1.1	3.0	6.0	3.5	20.0	2.4	1.7	3.6	0.2	0.0	1.6	2.3	1.8	14.3	20.0	5.1	3.3
310 B	H	2.4	2.9	2.7	4.9	20.0	6.8	4.4	4.8	3.1	15.0	3.4	0.0	2.3	4.6	7.0	1.8	1.6	3.8	20.0	20.0
311 B	Q	0.0	2.2	1.3	0.7	2.1	3.3	2.4	12.6	0.6	0.9	2.3	0.6	3.2	0.4	0.8	0.2	1.6	18.8	4.6	2.0
312 B	N	0.0	1.0	0.2	0.3	6.0	5.4	2.3	12.0	2.1	2.9	1.6	0.9	50.0	1.3	5.7	0.1	5.6	3.8	8.0	7.8
313 B	W	5.3	6.6	7.3	5.4	0.0	11.4	6.2	20.0	4.0	5.2	4.3	8.0	50.0	6.5	8.9	6.6	17.2	20.0	2.1	0.9
314 B	L	1.7	2.2	3.1	0.0	6.4	5.6	1.5	2.1	0.6	0.2	1.1	1.9	50.0	0.8	1.0	1.7	0.9	3.1	3.7	11.3
315 B	D	1.4	2.3	2.4	0.7	6.0	5.5	2.3	4.8	2.2	1.0	2.9	1.8	50.0	1.0	2.2	0.2	0.0	4.5	8.5	6.8
316 B	G	50.0	50.0	50.0	50.0	50.0	0.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
317 B	K	0.9	2.3	4.3	2.8	1.2	4.0	0.6	13.9	0.0	4.8	1.6	1.3	50.0	4.2	0.9	0.4	13.8	10.1	20.0	1.7
318 B	E	0.7	1.2	3.1	1.0	7.0	5.1	8.2	0.4	1.0	5.7	1.7	2.3	3.8	1.0	1.6	0.4	0.0	1.0	3.8	7.7
319 B	Y	6.5	7.1	8.5	8.8	0.0	12.5	3.9	3.1	5.2	5.4	8.4	7.2	50.0	9.0	13.7	7.2	5.8	3.9	20.0	1.7
320 B	K	3.1	4.3	7.3	4.3	20.0	8.6	15.0	1.4	0.0	11.6	3.6	6.6	50.0	2.9	2.4	4.0	3.3	2.0	20.0	20.0
321	C	0.0	6.5	20.	20.	20.	6.6	20.	20.	20.	20.	20.	20.	19.	20.	20.	3.1	11.	20.	20.	20.

B				0	0	0		0	0	0	0	0	0	7	0	0		2	0	0	0
322 B	K	2.3	3.2	3.5	1.8	20.0	7.9	20.0	1.1	0.6	4.9	3.7	2.2	50.0	0.9	0.3	3.3	1.6	0.0	20.0	20.0
323 B	V	4.0	4.6	6.9	8.1	20.0	10.6	20.0	9.0	17.1	7.9	8.1	10.5	50.0	8.7	20.0	5.6	4.6	0.0	20.0	20.0
324 B	S	1.3	3.0	1.4	0.0	2.1	6.0	4.4	1.3	2.4	0.6	2.7	2.2	50.0	1.3	3.6	1.5	1.6	1.8	3.6	2.4
325 B	N	3.4	5.1	9.0	4.7	20.0	8.2	20.0	16.6	16.6	20.0	20.0	0.0	50.0	6.3	20.0	4.6	8.8	17.8	20.0	20.0
326 B	K	0.3	2.1	2.0	0.9	1.0	3.5	2.0	2.9	0.9	2.9	2.8	0.1	4.4	0.0	1.1	0.1	3.2	2.1	5.2	0.7
327 B	A	1.9	3.3	4.7	3.5	20.0	7.0	20.0	20.0	0.3	0.0	1.9	1.9	20.0	3.0	2.3	3.3	20.0	20.0	20.0	20.0
328 B	L	3.7	3.6	3.8	4.4	50.0	8.4	7.0	50.0	3.8	0.0	2.6	4.0	50.0	4.2	8.7	4.8	2.9	12.3	50.0	50.0
329 B	P	3.3	8.5	20.0	20.0	50.0	8.0	16.5	50.0	18.5	20.0	14.7	20.0	0.0	20.0	20.0	1.4	17.1	16.4	50.0	50.0
330 B	A	0.5	2.0	2.8	0.5	2.4	3.9	1.2	4.0	0.0	2.0	2.1	0.8	20.0	0.0	0.5	0.8	0.2	4.6	8.2	2.6
331 B	P	1.7	3.8	6.4	10.1	20.0	4.7	11.0	10.1	7.5	20.0	5.5	5.0	0.0	7.6	7.4	2.6	20.0	10.1	17.6	20.0
332 B	I	1.7	2.9	1.3	1.7	14.8	7.0	13.9	1.7	3.1	0.0	1.7	1.7	50.0	1.8	5.3	2.0	1.9	3.4	20.0	20.0
333 B	E	1.9	2.5	1.9	0.0	8.9	5.9	8.2	1.2	3.0	6.4	3.4	2.0	3.1	1.1	2.3	1.8	1.6	1.6	8.9	9.3
334 B	K	2.9	3.9	3.7	2.6	20.0	8.3	12.1	1.5	2.6	5.3	3.7	4.3	50.0	1.9	0.0	3.4	1.8	1.4	9.9	20.0
335 B	T	0.0	2.1	7.2	7.0	4.2	0.4	3.3	17.3	6.5	7.7	5.2	5.5	3.5	7.0	5.7	0.2	5.5	11.5	5.2	3.1
336 B	I	0.5	1.6	2.1	0.7	20.0	5.0	6.1	0.0	1.3	5.3	2.1	1.8	20.0	0.6	3.1	1.1	0.8	0.7	19.4	20.0
337 B	S	1.1	2.1	4.0	2.0	3.1	3.2	2.0	50.0	0.0	1.6	0.9	1.9	15.8	1.1	2.2	1.4	50.0	50.0	5.5	3.9
338 B	K	0.6	2.3	3.0	3.0	9.4	5.3	10.6	2.2	1.1	0.0	3.2	1.5	16.2	2.7	2.7	1.1	2.8	3.5	8.1	11.0
339 B	A	1.1	2.4	1.2	0.8	4.3	3.6	3.7	2.6	1.8	2.6	3.5	0.0	2.3	0.5	1.3	0.2	0.8	2.0	6.7	3.8
340	K	0.9	2.0	1.4	0.8	3.0	3.4	2.9	2.1	0.8	2.5	2.3	0.2	1.0	0.1	1.2	0.0	0.5	2.0	5.5	3.2

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B

SPA™ technology; 1IIS template structure; - carbohydrate, no floated positions

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Table 56

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
239 A	S	0.2	4.6	2.7	0.0	20.0	4.6	14.5	11.0	1.9	0.3	2.0	1.9	8.1	1.4	2.6	0.4	5.7	11.6	20.0	20.0
240 A	V	1.5	2.4	2.4	6.9	20.0	7.4	20.0	5.1	9.9	5.9	5.5	2.4	1.1	12.3	13.1	2.6	0.5	0.0	20.0	20.0
263 A	V	2.3	2.8	6.3	16.5	20.0	8.8	20.0	9.6	7.3	7.3	15.3	4.8	50.0	16.4	17.4	2.8	1.4	0.0	20.0	20.0
264 A	V	1.8	3.1	2.6	1.8	0.0	6.3	1.9	0.6	2.4	0.8	2.7	2.1	1.6	2.3	2.7	2.3	1.1	0.5	3.5	0.0
266 A	V	4.9	5.2	6.9	12.3	20.0	11.1	20.0	0.8	11.9	20.0	8.5	6.6	50.0	12.5	20.0	6.1	3.7	0.0	20.0	20.0
296 A	Y	3.4	2.7	1.1	0.0	50.0	0.7	50.0	5.0	3.6	3.5	4.2	0.9	50.0	0.9	2.9	2.2	5.3	5.5	16.1	18.4
299 A	T	0.7	3.2	9.9	10.4	20.0	6.2	20.0	10.7	6.7	20.0	4.1	12.9	50.0	5.9	11.8	0.0	2.5	8.2	13.3	20.0
325 A	N	2.5	3.5	7.7	2.5	20.0	8.0	20.0	0.0	6.1	20.0	7.8	1.2	12.8	0.8	20.0	2.7	0.0	1.0	20.0	20.0
328 A	L	6.1	6.3	7.1	4.2	50.0	8.8	20.0	50.0	4.6	0.0	7.2	6.1	50.0	4.0	8.3	6.7	50.0	50.0	20.0	50.0
330 A	A	0.9	1.8	1.2	0.0	2.5	4.0	2.9	1.7	1.2	1.6	2.8	0.0	20.0	0.4	1.0	0.2	0.5	1.7	6.2	2.9
332 A	I	1.9	3.8	4.6	1.3	5.1	7.1	1.8	3.4	0.2	0.0	2.6	3.8	20.0	0.6	2.4	2.3	2.5	4.2	20.0	5.6
239 B	S	1.0	2.4	3.5	2.0	6.7	5.6	2.9	3.1	0.3	0.0	1.9	2.1	50.0	1.5	1.8	1.4	1.4	5.2	20.0	4.2
240 B	V	0.3	2.4	6.9	11.7	20.0	6.6	20.0	8.3	12.3	20.0	14.2	7.4	0.0	13.4	20.0	1.3	1.9	0.9	20.0	20.0
263 B	V	2.4	3.9	4.5	12.5	20.0	9.3	20.0	15.8	17.1	2.1	20.0	5.3	50.0	13.8	20.0	3.9	2.2	0.0	20.0	20.0
264 B	V	2.2	3.2	4.8	2.7	7.4	6.9	6.0	0.0	1.9	1.9	3.8	3.7	9.9	3.1	2.2	2.7	2.4	0.9	14.7	18.2
266 B	V	5.4	5.5	7.5	13.2	20.0	12.1	20.0	2.6	20.0	20.0	20.0	5.4	50.0	16.1	20.0	6.0	4.7	0.0	50.0	50.0
296 B	Y	1.5	2.7	1.3	1.2	4.0	4.1	3.6	1.1	1.9	2.6	3.5	0.0	20.0	0.7	1.8	1.1	1.4	1.3	6.5	4.2
299	T	0.0	2.2	7.5	10.	20.	4.8	20.	7.7	5.8	20.	10.	5.1	50.	10.	18.	0.3	1.1	5.4	20.	20.

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B					2	0		0			0	3		0	2	4				0	0
325 B	N	3.4	5.1	8.6	5.0	20.0	8.2	20.0	16.7	20.0	20.0	20.0	0.0	19.7	6.3	20.0	4.6	8.6	18.2	20.0	20.0
328 B	L	3.6	3.5	3.8	3.9	50.0	8.3	7.0	50.0	2.9	0.0	1.9	3.8	50.0	3.4	8.4	4.7	2.9	12.5	50.0	50.0
330 B	A	0.7	2.1	2.9	0.7	2.7	4.0	1.4	4.8	0.0	2.2	2.3	0.8	20.0	0.2	0.8	1.1	0.2	4.7	7.8	3.2
332 B	I	1.8	2.9	1.2	1.8	13.5	7.0	9.9	1.7	3.2	0.0	1.7	1.9	50.0	1.2	5.4	2.0	2.0	3.3	20.0	20.0

SPA™ technology; D129G 1IIS template structure; + carbohydrate

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Table 57

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
239 A	S	1.2	3.5	1.7	0.0	20.0	5.8	11.0	6.6	2.9	3.9	3.9	2.7	8.5	1.3	2.7	0.6	3.5	5.4	20.0	20.0
240 A	V	1.2	2.4	6.0	14.0	20.0	7.1	20.0	6.7	9.4	10.1	7.5	4.4	1.8	14.8	20.0	2.0	0.4	0.0	20.0	20.0
263 A	V	0.0	0.4	1.0	8.7	20.0	6.9	4.4	11.7	4.9	16.0	19.2	0.8	50.0	11.7	20.0	1.4	0.1	1.0	20.0	20.0
264 A	V	2.9	3.7	6.3	2.8	11.6	7.6	13.2	0.0	3.2	3.4	4.1	4.2	7.1	2.9	3.4	3.1	1.9	0.8	12.8	16.3
266 A	V	4.8	5.9	6.8	9.5	50.0	10.3	20.0	3.5	12.7	12.2	12.7	4.1	50.0	11.9	11.9	5.2	2.9	0.0	50.0	50.0
296 A	Y	0.8	2.0	1.5	0.1	0.2	3.4	1.5	6.6	1.7	0.6	1.8	1.2	2.6	0.0	1.6	0.2	2.5	5.6	3.8	0.0
299 A	T	1.9	3.7	7.5	0.0	20.0	7.9	14.2	2.9	0.8	3.4	4.4	2.3	50.0	1.9	3.0	3.5	4.1	3.3	20.0	20.0
325 A	N	1.0	1.4	3.1	2.8	20.0	7.4	20.0	8.5	7.7	10.4	6.1	2.8	15.4	5.4	20.0	0.0	0.1	3.8	20.0	20.0
328 A	L	2.5	5.3	4.0	1.9	50.0	7.5	20.0	20.0	1.6	0.2	0.0	2.9	50.0	0.4	4.8	3.2	2.9	7.0	50.0	50.0
330 A	A	0.9	2.1	1.8	1.2	2.4	2.7	3.1	3.1	1.4	2.1	3.5	0.5	20.0	0.8	1.0	0.0	0.5	2.9	5.2	2.9
332 A	I	2.9	3.7	3.9	0.9	6.1	7.8	2.5	0.0	2.7	0.8	2.8	3.5	50.0	0.7	3.7	2.9	2.5	1.0	8.1	6.9
239 B	S	1.9	3.1	3.0	1.9	1.5	6.2	2.3	14.1	1.8	1.4	2.9	1.8	0.0	1.9	3.2	1.9	2.3	7.7	6.6	15.8
240 B	V	0.5	1.7	5.0	13.3	20.0	6.6	20.0	1.2	12.4	12.1	8.8	4.6	6.3	20.0	20.0	1.0	0.2	0.0	20.0	20.0
263 B	V	2.9	3.2	6.4	18.2	10.1	9.2	6.9	12.8	6.0	20.0	10.3	5.7	50.0	17.5	20.0	3.2	2.2	0.0	20.0	20.0
264 B	V	2.9	3.6	4.4	3.0	8.8	7.1	6.2	0.0	2.3	1.9	4.5	3.4	1.7	3.2	3.5	3.5	2.0	0.9	12.0	16.4
266 B	V	4.4	4.6	2.6	6.6	20.0	10.7	20.0	0.0	4.9	1.7	8.5	5.6	50.0	6.0	12.4	5.3	4.6	1.5	20.0	50.0
296 B	Y	0.0	7.1	6.7	7.2	20.0	0.1	18.6	50.0	7.0	2.7	6.6	6.8	50.0	7.2	9.3	2.3	50.0	50.0	20.0	14.1
299	T	0.0	3.2	10.	6.0	20.	5.5	20.	15.	3.2	5.9	4.4	6.4	50.	5.7	9.4	1.2	1.4	13.	20.	20.

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B				4		0		0	9					0					7	0	0
325 B	N	1.4	2.5	5.0	0.0	20.0	7.0	20.0	20.0	1.0	2.2	1.0	0.3	1.9	1.1	20.0	2.6	5.1	20.0	20.0	20.0
328 B	L	0.4	1.3	5.6	0.0	50.0	4.5	50.0	50.0	1.9	2.4	2.4	8.3	50.0	0.8	16.4	1.0	1.2	50.0	50.0	50.0
330 B	A	0.6	1.4	2.5	0.9	3.1	2.5	1.2	20.0	0.0	2.4	2.1	0.3	20.0	0.4	0.6	0.0	4.0	20.0	13.5	3.4
332 B	I	4.3	5.3	5.7	0.0	11.4	9.3	4.3	2.5	5.8	2.0	4.0	6.5	17.9	3.7	5.9	4.6	4.2	3.7	20.0	11.6

SPA™ technology; D129G 1IIX template structure; + carbohydrate

Table 58

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
239 A	S	1.2	2.3	2.2	1.8	7.9	5.5	7.6	0.5	0.2	1.8	2.6	1.4	0.9	1.3	1.9	1.5	0.8	0.0	8.6	9.6
240 A	V	0.7	2.9	6.8	4.3	20.0	6.5	20.0	0.0	10.7	20.0	3.1	9.1	2.1	7.7	20.0	1.4	1.1	2.4	20.0	20.0
263 A	V	1.7	2.9	4.6	18.8	20.0	8.4	5.8	15.1	2.3	14.5	2.1	3.2	50.0	20.0	15.0	3.6	1.2	0.0	20.0	20.0
264 A	V	2.7	3.3	3.6	1.5	13.9	6.7	5.9	0.0	2.3	4.9	3.7	3.2	1.9	2.5	3.0	3.0	2.5	0.7	19.9	19.0
266 A	V	3.5	3.5	5.7	12.4	20.0	10.0	20.0	5.7	6.3	7.8	7.4	5.2	50.0	16.6	20.0	4.2	1.7	0.0	20.0	50.0
296 A	Y	2.6	50.0	50.0	50.0	50.0	0.0	50.0	50.0	18.5	18.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	13.6
299 A	T	0.2	0.7	6.6	1.2	20.0	5.6	9.6	1.6	0.8	1.5	1.8	4.8	50.0	1.0	9.2	0.0	0.0	1.6	20.0	20.0
325 A	N	3.1	3.6	7.3	2.4	20.0	7.7	20.0	20.0	20.0	10.0	13.1	3.6	50.0	0.0	20.0	4.0	9.7	20.0	20.0	20.0
328 A	L	0.6	0.0	1.5	5.4	50.0	1.6	50.0	50.0	3.1	4.2	9.6	1.4	50.0	6.9	9.6	0.6	0.1	50.0	50.0	50.0
330 A	A	1.9	2.5	4.1	2.8	4.5	4.1	3.0	3.2	1.0	2.7	3.5	2.1	20.0	2.4	2.6	1.3	0.0	3.9	7.6	5.3
332 A	I	2.3	3.5	2.2	0.8	20.0	6.8	9.6	0.0	3.4	0.2	2.6	2.8	14.5	3.3	4.6	2.6	1.3	0.9	10.5	20.0
239 B	S	1.4	3.6	2.5	1.4	16.8	5.8	6.2	5.0	2.5	1.4	2.0	3.8	0.3	0.5	2.4	0.0	1.6	5.3	20.0	19.5
240 B	V	0.0	2.6	12.8	18.6	20.0	5.7	20.0	12.7	10.4	20.0	8.5	15.1	3.1	20.0	20.0	1.0	0.2	2.4	20.0	20.0
263 B	V	1.1	2.4	3.6	20.0	20.0	7.8	17.7	11.8	4.5	20.0	6.3	3.3	50.0	20.0	20.0	3.2	1.2	0.0	20.0	20.0
264 B	V	3.3	4.0	5.0	2.9	14.2	7.5	4.8	0.0	2.6	3.6	4.6	3.5	1.7	3.1	4.1	3.9	2.9	1.3	6.9	20.0
266 B	V	2.9	3.3	4.9	11.3	50.0	9.5	20.0	20.0	20.0	7.9	15.0	4.5	50.0	4.9	20.0	1.9	0.0	3.6	50.0	50.0
296 B	Y	2.8	50.0	50.0	50.0	50.0	0.0	50.0	50.0	17.7	18.7	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	11.3
299	T	0.0	3.8	12.	9.2	20.	5.9	20.	7.3	4.8	3.2	4.3	8.0	50.	12.	8.8	0.2	2.1	4.4	20.	20.

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B				6		0		0						0	3				0	0
325 B	N	0.3	2.0	5.5	2.2	50.0	6.1	20.0	0.0	10.5	15.5	14.6	1.3	10.0	2.4	20.0	2.3	2.0	1.0	20.0	50.0
328 B	L	5.4	5.7	7.3	4.4	50.0	9.8	20.0	50.0	2.5	0.0	5.1	5.9	50.0	2.8	7.4	6.1	6.4	50.0	50.0	50.0
330 B	A	0.6	1.4	3.2	1.3	3.9	3.2	2.7	4.0	1.3	3.7	3.1	0.7	20.0	0.6	1.3	0.0	0.4	4.2	8.2	3.6
332 B	I	1.9	3.1	2.7	1.7	5.2	6.9	3.1	0.4	1.3	0.0	1.9	2.6	7.7	1.3	2.2	2.3	1.6	2.0	10.4	5.6

SPA™ technology; D129G 1E4K template structure; + carbohydrate

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Table 59

Pos	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
239 A	S	1.4	2.6	3.1	1.0	20.0	5.7	4.8	3.4	2.0	1.2	2.6	1.6	4.8	0.0	2.1	1.3	2.1	3.3	13.8	19.6
240 A	V	2.9	3.5	3.7	4.6	20.0	8.2	10.8	0.0	9.1	3.2	5.4	3.1	4.8	5.5	17.5	4.0	1.8	1.2	20.0	20.0
263 A	V	3.6	4.9	6.2	8.7	20.0	9.9	20.0	3.7	4.2	0.5	6.7	6.1	50.0	9.5	20.0	5.1	3.6	0.0	20.0	20.0
264 A	V	1.8	2.8	3.3	2.0	2.9	6.2	3.1	0.0	2.4	0.8	3.0	2.4	6.1	1.4	2.8	2.4	1.9	0.8	10.2	2.2
266 A	V	4.4	5.2	4.9	7.1	20.0	10.6	20.0	1.0	12.1	4.8	9.1	4.6	50.0	7.9	12.6	5.8	3.5	0.0	20.0	20.0
296 A	Y	1.2	2.9	0.7	1.4	3.1	3.9	2.7	2.4	2.3	1.9	2.2	0.0	1.6	1.4	3.0	0.9	1.0	3.5	6.0	2.6
299 A	T	0.0	2.6	6.0	11.5	20.0	5.3	20.0	20.0	6.0	20.0	4.4	3.0	50.0	14.1	13.2	0.9	3.8	15.1	15.0	20.0
325 A	N	5.2	7.0	6.6	6.9	50.0	11.3	20.0	1.3	14.3	13.5	13.9	0.0	5.0	6.0	20.0	6.0	4.6	3.2	20.0	50.0
328 A	L	4.8	5.5	7.0	3.2	20.0	10.5	20.0	50.0	5.1	0.0	8.5	5.5	50.0	3.5	8.2	5.5	13.4	50.0	20.0	50.0
330 A	A	0.9	1.8	1.1	0.9	3.5	4.0	3.0	2.3	1.2	1.6	2.8	0.0	14.5	0.9	1.1	0.1	0.4	2.0	6.4	3.2
332 A	I	5.3	6.4	6.7	4.8	8.2	9.9	5.2	3.1	0.0	3.6	5.2	6.8	20.0	3.5	4.6	5.5	4.8	4.0	11.2	7.1
239 B	S	0.7	2.3	2.6	2.0	5.3	5.1	3.3	1.7	0.0	0.0	2.0	0.8	15.5	0.9	0.8	0.7	0.7	3.3	8.2	6.0
240 B	V	2.3	3.0	4.1	7.3	20.0	8.1	20.0	5.1	20.0	11.8	10.9	3.8	2.0	17.0	20.0	3.6	1.3	0.0	20.0	20.0
263 B	V	3.2	4.3	7.3	8.3	20.0	9.6	20.0	13.3	8.5	0.6	20.0	6.0	50.0	8.5	20.0	4.6	4.0	0.0	20.0	20.0
264 B	V	2.1	3.2	3.7	2.7	17.8	6.6	11.5	0.0	2.0	0.8	3.5	3.0	7.8	2.0	1.5	2.5	1.3	1.0	13.9	20.0
266 B	V	5.0	5.0	5.2	16.3	20.0	11.2	20.0	2.3	20.0	14.3	17.3	2.5	50.0	11.6	20.0	5.4	3.9	0.0	20.0	20.0
296 B	Y	0.9	2.3	1.0	0.5	2.7	3.7	2.5	1.2	1.3	2.1	3.0	0.0	7.0	0.4	1.1	0.3	0.8	1.8	6.0	2.4
299	T	1.1	2.2	7.6	5.4	20.	6.4	12.	1.8	3.9	17.	6.9	3.9	20.	4.6	10.	0.8	0.0	1.9	20.	20.

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B						0		8			5			0		3				0	0
325 B	N	10.1	11.5	13.1	11.2	20.0	15.7	20.0	8.6	14.3	17.1	20.0	0.0	16.1	10.6	20.0	11.1	10.9	10.5	20.0	20.0
328 B	L	2.9	4.1	4.8	3.5	50.0	8.5	1.7	9.6	1.5	0.0	1.5	3.5	50.0	3.3	2.0	3.3	1.9	5.2	50.0	50.0
330 B	A	0.1	2.0	1.4	1.8	1.6	4.0	3.0	2.0	0.5	0.5	2.6	0.0	20.0	0.7	2.0	0.3	0.6	2.1	4.4	2.4
332 B	I	3.4	4.4	3.5	3.1	6.1	8.2	4.1	0.0	3.3	1.3	3.3	4.0	15.7	0.8	2.1	3.9	2.7	1.1	20.0	6.1

SPA™ technology; Fc/Fc□RIIb model template structure; - carbohydrate

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[202] The results of the design calculations presented above in Tables 1 – 59 were used to construct a series of Fc variant libraries for experimental production and screening. Experimental libraries were designed in successive rounds of computational and experimental screening. Design of subsequent Fc libraries benefitted from feedback from prior libraries, and thus typically comprised combinations of Fc variants that showed favorable properties in the previous screen. The entire set of Fc variants that were constructed and experimentally tested is shown in Table 60. In this table, row 1 lists the variable positions, and the rows that follow indicate the amino acids at those variable positions for WT and the Fc variants. For example, variant 18 has the following four mutations: F241E, F243Y, V262T, and V264R. The variable position residues that compose this set of Fc variants are illustrated structurally in Figure 3, and are presented in the context of the human IgG1 Fc sequence in Figure 4.

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Table 60

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
1												A																		
2												L																		
3												I																		
4					W																									
5					L																									
6						W																								
7						L																								
8					L	L				I		I																		
9					W	W																								
10					W	W				A		A																		
11					L					I																				
12						L						I																		
13						L				I		W																		
14					Y	Y				T		T																		
15					E	R				E		R																		
16					E	Q				T		E																		
17					R	Q				T		R																		
18					E	Y				T		R																		
19																									M					
20																									E					
21																									F					
22																												E		
23																									M			E		
24							H																							
25								A																						
26									V																					
27																					F									
28							H	A	V																					
29									G																					
30												I																E		

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313

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
31					E	R				E		R																E		
32					E	Q				T		E																E		
33					R	Q				T		R																E		
34					E	Y				T		R																E		
35																			A											
36																			A									E		
37																			A										A	A
41			E																									E		
42			Q																									E		
43			E																											
44													G																	
45													N																	
46			E										G																	
47			E										N																	
48			E										Q																	
49																	E													
50																	Q													
51																			T											
52																			N											
53																				I										
54																								S						
55																								N						
56															Q									S						
57															L									S						
58																								L						
59																										F				
60																											L			
61																											Y			
62																												D		

121

314

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
62																												D		
63																		S												
64																		D												
65																		S										E		
66																		D										E		
67																		E										E		
68													Y					D										E		
69													Y					D										E		
70													F					E										E		
71																									I			E		
72																									Q			E		
73																												N		
74																												Q		
75												T																		
76												F																		
77				I																										
78											I																			
79														I																
80																				A										
81																				S										
82																				V										
83																						Q								
84																						L								
85																						I								
86			D																											
87			N																											
88			F																											
89			D																									D		
90			D																									E		
91			D																									N		
92			D																									Q		

122

315

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
93			E																									D		
94			E																									N		
95			E																									Q		
96			N																									D		
97			N																									E		
98			N																									N		
99			N																									Q		
100			Q																									D		
101			Q																									N		
102			Q																									Q		
103																							E							
104																	D													
105																	N													
106					Y	Y				T		T						D										E		
107																											Y	E		
108												I															Y	E		
109																											L	E		
110												I															L	E		
111	D																													
112	E																													
112	N																													
114	Q																													
115	T																													
116	H																													
117	Y																													
118	I																													
119	V																													
120	F																													
121		D																												
122		S																												

123

316

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
123		N																												
124		Q																												
125		T																												
126		H																												
127		Y																												
128		I																												
129		V																												
130		F																												
131			T																											
132			H																											
133			Y																											
134				A																										
135				T																										
136				M																										
137											A																			
138											T																			
139											M																			
140												M																		
141												Y																		
142														A																
143														T																
144														M																
145																H														
146																Y														
147																F														
148																R														
149																	S													
150																	T													
151																	L													
152																	I													

124

317

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
153																			H											
154																				H										
155																											V			
156																											I			
157																											F			
158																											R			
159																											H			
160																						D								
161																						E								
162																						A								
163																						T								
164																						V								
165																						H								
166																									D			E		
167																									E			E		
168																									N			E		
169																									Q			E		
170																									V			E		
171																									T			E		
172																									H			E		
173																									I			E		
174																									A					
175																												T		
176																												H		
177																												Y		
178																												A		
179			E									I																E		
180			Q									I																E		
181			E									I															Y	E		
182			E									I							A								Y	E		

125

318

Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
183			D															D										E		
184			E															D										E		
185			D										V					D										E		
186			D										I					D										E		
187			D										L					D										E		
188			D										F					D										E		
189			D										Y					D										E		
190			D										H					D										E		
191			D										T					D										E		
192												E						D										E		
193																	D	D										E		
194																	E	D										E		
195																	N	D										E		
196																	Q	D										E		
197																	H	D										E		
198																	T	D										E		
199																		D		V								E		
200																		D		I								E		
201																		D		L								E		
202																		D		F								E		
203																		D		H								E		
204																		D		E								E		
205																		D									Y	E		
206																		D	A								Y	E		
207			D																								Y	E		
208			N																								Y	E		
209			D																								L	E		
210			N																								L	E		
211												I							A									E		
212			D																A									E		

126

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Table 60 (continued)

Position	234	235	239	240	241	243	244	245	247	262	263	264	265	266	267	269	296	297	298	299	313	325	326	327	328	329	330	332	333	334
WT	L	L	S	V	F	F	P	P	P	V	V	V	D	V	S	E	Y	N	S	T	W	N	K	A	L	P	A	I	E	K
213			N																A									E		
214			D									I																E		
215			D									I							A									E		
216			D									I															L	E		

[203] Example 2: Experimental production and screening of Fc libraries

The majority of experimentation on the Fc variants was carried out in the context of the anti-cancer antibody alemtuzumab (Campath®, a registered trademark of Ilex Pharmaceuticals LP). Alemtuzumab binds a short linear epitope within its target antigen CD52 (Hale *et al.*, 1990, *Tissue Antigens* 35:118-127; Hale, 1995, *Immunotechnology* 1:175-187). Alemtuzumab has been chosen as the primary engineering template because its efficacy is due in part to its ability to recruit effector cells (Dyer *et al.*, 1989, *Blood* 73:1431-1439; Friend *et al.*, 1991, *Transplant Proc* 23:2253-2254; Hale *et al.*, 1998, *Blood* 92:4581-4590; Glennie *et al.*, 2000, *Immunol Today* 21:403-410), and because production and use of its antigen in binding assays are relatively straightforward. In order to evaluate the optimized Fc variants of the present invention in the context of other antibodies, select Fc variants were evaluated in the anti-CD20 antibody rituximab (Rituxan®, a registered trademark of IDEC Pharmaceuticals Corporation), and the anti-Her2 antibody trastuzumab (Herceptin®, a registered trademark of Genentech). The use of alemtuzumab, rituximab, and trastuzumab for screening purposes is not meant to constrain the present invention to any particular antibody.

[204] The IgG1 full length light (V_L-C_L) and heavy (V_H-Cγ1-Cγ2-Cγ3) chain antibody genes for alemtuzumab, rituximab, and trastuzumab were constructed with convenient end restriction sites to facilitate subcloning. The genes were ligated into the mammalian expression vector pcDNA3.1Zeo (Invitrogen). The V_H-Cγ1-Cγ2-Cγ3 clone in pcDNA3.1zeo was used as a template for mutagenesis of the Fc region. Mutations were introduced into this clone using PCR-based mutagenesis techniques. Fc variants were sequenced to confirm the fidelity of the sequence. Plasmids containing heavy chain gene (V_H-Cγ1-Cγ2-Cγ3) (wild-type or variants) were co-transfected with plasmid containing light chain gene (V_L-C_L) into 293T cells. Media were harvested 5 days after transfection. Expression of immunoglobulin was monitored by screening the culture supernatant of transfectomas by western using peroxidase-conjugated goat-anti human IgG (Jackson ImmunoResearch, catalog # 109-035-088). Figure 6 shows expression of wild-type alemtuzumab and variants 1 through 10 in 293T cells. Antibodies were purified from the supernatant using protein A affinity chromatography (Pierce, Catalog # 20334. Figure 7 shows results of the protein purification for WT alemtuzumab. Antibody Fc variants showed similar expression and purification results to WT. Some Fc variants were deglycosylated in order to determine their solution and functional properties in the absence of carbohydrate. To obtain deglycosylated antibodies, purified alemtuzumab antibodies were incubated with peptide-N-glycosidase (PNGase F) at 37°C for 24h. Figure 8 presents an SDS PAGE gel confirming deglycosylation for several Fc variants and WT alemtuzumab.

[205] In order to confirm the functional fidelity of alemtuzumab produced under these conditions, the antigenic CD52 peptide, fused to GST, was expressed in E.coli BL21 (DE3) under IPTG induction. Both un-induced and induced samples were run on a SDS PAGE gel, and transferred to PVDF membrane. For western analysis, either alemtuzumab from Sotec (final concentration 2.5ng/ul) or

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media of transfected 293T cells (final alemtuzumab concentration about 0.1-0.2ng/ul) were used as primary antibody, and peroxidase-conjugated goat-anti human IgG was used as secondary antibody. Figure 9 presents these results. The ability to bind target antigen confirms the structural and functional fidelity of the expressed alemtuzumab. Fc variants that have the same variable region as WT alemtuzumab are anticipated to maintain a comparable binding affinity for antigen.

[206] In order to screen for Fc/FcγR binding, the extracellular regions of human V158 FcγRIIIa, human F158 FcγRIIIa, human FcγRIIb, human FcγRIIa, and mouse FcγRIII, were expressed and purified. Figure 10 presents an SDS PAGE gel that shows the results of expression and purification of human V158 FcγRIIIa. The extracellular region of this receptor was obtained by PCR from a clone obtained from the Mammalian Gene Collection (MGC:22630). The receptor was fused with glutathione S-Transferase (GST) to enable screening. Tagged Fc□RIIIa was transfected in 293T cells, and media containing secreted Fc□RIIIa were harvested 3 days later and purified. For western analysis, membrane was probed with anti-GST antibody.

[207] Binding affinity to Fc□RIIIa and FcγRIIb was measured for all designed Fc variants using an AlphaScreen™ assay (Amplified Luminescent Proximity Homogeneous Assay (ALPHA), PerkinElmer, Wellesley, MA), a bead-based non-radioactive luminescent proximity assay. Laser excitation of a donor bead excites oxygen, which if sufficiently close to the acceptor bead generates a cascade of chemiluminescent events, ultimately leading to fluorescence emission at 520-620 nm. The AlphaScreen™ assay was applied as a competition assay for screening Fc variants. WT alemtuzumab antibody was biotinylated by standard methods for attachment to streptavidin donor beads, and GST-tagged FcγR was bound to glutathione chelate acceptor beads. In the absence of competing Fc variants, WT antibody and FcγR interact and produce a signal at 520-620 nm. Addition of untagged Fc variant competes with the WT Fc/FcγR interaction, reducing fluorescence quantitatively to enable determination of relative binding affinities. All Fc variants were screened for V158 FcγRIIIa binding using the AlphaScreen™ assay. Select Fc variants were subsequently screened for binding to FcγRIIb, as well as other FcγRs and Fc ligands.

[208] Figure 11 shows AlphaScreen™ data for binding to human V158 FcγRIIIa by select Fc variants. The binding data were normalized to the maximum and minimum luminescence signal provided by the baselines at low and high concentrations of competitor antibody respectively. The data were fit to a one site competition model using nonlinear regression, and these fits are represented by the curves in the figure. These fits provide the inhibitory concentration 50% (IC50) (i.e. the concentration required for 50% inhibition) for each antibody, illustrated by the dotted lines in Figure 11, thus enabling the relative binding affinities of Fc variants to be quantitatively determined. Here, WT alemtuzumab has an IC50 of $(4.63 \times 10^{-9}) \times (2) = 9.2$ nM, whereas S239D has an IC50 of $(3.98 \times 10^{-10}) \times (2) = 0.8$ nM. Thus S239D alemtuzumab binds 9.2 nM / 0.8 nM = 11.64-fold more tightly

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than WT alemtuzumab to human V158 FcγRIIIa. Similar calculations were performed for the binding of all Fc variants to human V158 FcγRIIIa. Select Fc variants were also screened for binding to human Fc□RIIb, and examples of these AlphaScreen™ binding data are shown in Figure 12. Table 61 presents the fold-enhancement or fold-reduction relative to the parent antibody for binding of Fc variants to human V158 FcγRIIIa (column 3) and human FcγRIIb (column 4), as determined by the AlphaScreen™ assay. For these data, a fold above 1 indicates an enhancement in binding affinity, and a fold below 1 indicates a reduction in binding affinity relative to WT Fc. All data were obtained in the context of alemtuzumab, except for those indicated with an asterix (*), which were tested in the context of trastuzumab.

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Table 61

Variant	Substitution(s)	FcγRIIIa Fold	FcγRIIb Fold	FcγIIIa-fold : FcγIIb-fold
1	V264A	0.53		
2	V264L	0.56		
3	V264I	1.43		
4	F241W	0.29		
5	F241L	0.26		
6	F243W	0.51		
7	F243L	0.51		
8	F241L/F243L/V262I/V264I	0.09		
9	F241W/F243W	0.07		
10	F241W/F243W/V262A/V264A	0.04		
11	F241L/V262I	0.06		
12	F243L/V264I	1.23		
13	F243L/V262I/V264W	0.02		
14	F241Y/F243Y/V262T/V264T	0.05		
15	F241E/F243R/V262E/V264R	0.05		
16	F241E/F243Q/V262T/V264E	0.07		
17	F241R/F243Q/V262T/V264R	0.02		
18	F241E/F243Y/V262T/V264R	0.05		
19	L328M	0.21		
20	L328E	0.12		
21	L328F	0.24		
22	I332E	6.72	3.93	1.71
23	L328M/I332E	2.60		
24	P244H	0.83		
25	P245A	0.25		
26	P247V	0.53		
27	W313F	0.88		
28	P244H/P245A/P247V	0.93		
29	P247G	0.54		
30	V264I/I332E	12.49	1.57*	7.96
31	F241E/F243R/V262E/V264R/I332E	0.19		
32	F241E/F243Q/V262T/V264E/I332E			
33	F241R/F243Q/V262T/V264R/I332E			
34	F241E/F243Y/V262T/V264R/I332E	0.10		
35	S298A	2.21		
36	S298A/I332E	21.73		
37	S298A/E333A/K334A	2.56		
41	S239E/I332E	5.80	3.49	1.66
42	S239Q/I332E	6.60	4.68	1.41
43	S239E	10.16		
44	D265G	<0.02		
45	D265N	<0.02		
46	S239E/D265G	<0.02		
47	S239E/D265N	0.02		
48	S239E/D265Q	0.05		
49	Y296E	0.73	1.11	0.66
50	Y296Q	0.52	0.43	1.21

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Table 61 (continued)

Variant	Substitution(s)	FcγRIIIa Fold	FcγRIIb Fold	FcγIIIa-fold : FcγIIb-fold
51	S298T	0.94	<0.02	
52	S298N	0.41	<0.02	
53	T299I	<0.02		
54	A327S	0.23	0.39	0.59
55	A327N	0.19	1.15	0.17
56	S267Q/A327S	0.03		
57	S267L/A327S	<0.02		
58	A327L	0.05		
59	P329F	<0.02		
60	A330L	0.73	0.38	1.92
61	A330Y	1.64	0.75	2.19
62	I332D	17.80	3.34	5.33
63	N297S	<0.02		
64	N297D	<0.02		
65	N297S/I332E	<0.02		
66	N297D/I332E	0.08	<0.02	
67	N297E/I332E	<0.02		
68	D265Y/N297D/I332E	<0.02		
69	D265Y/N297D/T299L/I332E	<0.02		
70	D265F/N297E/I332E	<0.02		
71	L328I/I332E	7.03		
72	L328Q/I332E	1.54		
73	I332N	0.39		
74	I332Q	0.37		
75	V264T	2.73		
76	V264F	0.16		
77	V240I	3.25		
78	V263I	0.10		
79	V266I	1.86		
80	T299A	0.03		
81	T299S	0.15		
82	T299V	<0.02		
83	N325Q	<0.02		
84	N325L	<0.02		
85	N325I	<0.02		
86	S239D	11.64	4.47*	2.60
87	S239N	<0.02		
88	S239F	0.22	<0.02	
89	S239D/I332D	14.10		
90	S239D/I332E	56.10	19.71*	2.85
91	S239D/I332N	7.19		
92	S239D/I332Q	9.28		
93	S239E/I332D	9.33		
94	S239E/I332N	11.93		
95	S239E/I332Q	3.80		
96	S239N/I332D	3.08		
97	S239N/I332E	14.21		
98	S239N/I332N	0.43		

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Table 61 (continued)

Variant	Substitution(s)	FcγRIIIa Fold	FcγRIIb Fold	FcγIIIa-fold : FcγIIb-fold
99	S239N/I332Q	0.56		
100	S239Q/I332D	5.05		
101	S239Q/I332N	0.39		
102	S239Q/I332Q	0.59		
103	K326E	3.85		
104	Y296D	0.62		
105	Y296N	0.29		
106	F241Y/F243Y/V262T/V264T/N297D/I332E	0.15		
107	A330Y/I332E	12.02	4.40	2.73
108	V264I/A330Y/I332E	12.00	3.54	3.39
109	A330L/I332E	10.34	2.03	5.09
110	V264I/A330L/I332E	11.15	1.79	6.23
111	L234D	0.21		
112	L234E	1.34	2.21	0.61
113	L234N	0.56	1.39	0.40
114	L234Q	0.37		
115	L234T	0.35		
116	L234H	0.33		
117	L234Y	1.42	1.08	1.31
118	L234I	1.55	1.14	1.36
119	L234V	0.38		
120	L234F	0.30		
121	L235D	1.66	3.63	0.46
122	L235S	1.25		
123	L235N	0.40		
124	L235Q	0.51		
125	L235T	0.52		
126	L235H	0.41		
127	L235Y	1.19	10.15	0.12
128	L235I	1.10	0.94	1.17
129	L235V	0.48		
130	L235F	0.73	3.53	0.21
131	S239T	1.34		
132	S239H	0.20		
133	S239Y	0.21		
134	V240A	0.70	0.14	5.00
135	V240T			
136	V240M	2.06	1.38	1.49
137	V263A			
138	V263T	0.43		
139	V263M	0.05		
140	V264M	0.26		
141	V264Y	1.02	0.27	3.78
142	V266A	<0.02		
143	V266T	0.45		
144	V266M	0.62		
145	E269H	<0.02		
146	E269Y	0.12		

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Table 61 (continued)

Variant	Substitution(s)	FcγRIIIa Fold	FcγRIIb Fold	FcγIIIa-fold : FcγIIb-fold
147	E269F	0.16		
148	E269R	0.05		
149	Y296S	0.12		
150	Y296T	<0.02		
151	Y296L	0.22		
152	Y296I	0.09		
153	A298H	0.27		
154	T299H	<0.02		
155	A330V	0.43		
156	A330I	1.71	0.02	85.5
157	A330F	0.60		
158	A330R	<0.02		
159	A330H	0.52		
160	N325D	0.41		
161	N325E	<0.02		
162	N325A	0.11		
163	N325T	1.10		
164	N325V	0.48		
165	N325H	0.73		
166	L328D/I332E	1.34		
167	L328E/I332E	0.20		
168	L328N/I332E	<0.02		
169	L328Q/I332E	0.70		
170	L328V/I332E	2.06		
171	L328T/I332E	1.10		
172	L328H/I332E	<0.02		
173	L328I/I332E	3.49		
174	L328A	0.20		
175	I332T	0.72		
176	I332H	0.46		
177	I332Y	0.76		
178	I332A	0.89		
179	S239E/V264I/I332E	15.46		
180	S239Q/V264I/I332E	2.14		
181	S239E/V264I/A330Y/I332E	8.53		
182	S239E/V264I/S298A/A330Y/I332E			
183	S239D/N297D/I332E	0.28		
184	S239E/N297D/I332E	0.06		
185	S239D/D265V/N297D/I332E			
186	S239D/D265I/N297D/I332E			
187	S239D/D265L/N297D/I332E	<0.02		
188	S239D/D265F/N297D/I332E	<0.02		
189	S239D/D265Y/N297D/I332E	0.02		
190	S239D/D265H/N297D/I332E	0.04		
191	S239D/D265T/N297D/I332E	<0.02		
192	V264I/N297D/I332E	0.05		
193	Y296D/N297D/I332E			
194	Y296E/N297D/I332E	<0.02		

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Table 61 (continued)

Variant	Substitution(s)	FcγRIIIa Fold	FcγRIIb Fold	FcγIIIa-fold : FcγIIb-fold
195	Y296N/N297D/I332E	0.04		
196	Y296Q/N297D/I332E	<0.02		
197	Y296H/N297D/I332E	<0.02		
198	Y296T/N297D/I332E	<0.02		
199	N297D/T299V/I332E	<0.02		
200	N297D/T299I/I332E	<0.02		
201	N297D/T299L/I332E	<0.02		
202	N297D/T299F/I332E	<0.02		
203	N297D/T299H/I332E	<0.02		
204	N297D/T299E/I332E	<0.02		
205	N297D/A330Y/I332E	0.43		
206	N297D/S298A/A330Y/I332E			
207*	S239D/A330Y/I332E	129.58		
208*	S239N/A330Y/I332E	14.22		
209*	S239D/A330L/I332E	138.63	7.50	18.48
210*	S239N/A330L/I332E	12.95		
211*	V264I/S298A/I332E	16.50		
212*	S239D/S298A/I332E	295.16	6.16	47.92
213*	S239N/S298A/I332E	32.14	5.15	6.24
214*	S239D/V264I/I332E	36.58	14.39	2.54
215*	S239D/V264I/S298A/I332E			
216*	S239D/V264I/A330L/I332E			

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[209] Example 3: Selectively enhanced binding to FcγRs

A number of promising Fc variants with optimized properties were obtained from the FcγRIIIa and FcγRIIb screen. Table 61 provides Fc variants that bind more tightly to FcγRIIIa, and thus are candidates for improving the effector function of antibodies and Fc fusions. These include a number of variants that comprise substitutions at 239, 264, 330, and 332. Figure 13 shows AlphaScreen™ binding data for some of these Fc variants. The majority of these Fc variants provide substantially greater FcγRIIIa binding enhancements over S298A/E333A/K334A.

[210] Although the majority of Fc variants were screened in the context of the antibody alemtuzumab, select Fc variants were also screened in the context of rituximab and trastuzumab. AlphaScreen™ data for binding of select Fc variants to human V158 FcγRIIIa in the context of rituximab and trastuzumab are shown in Figures 14 and 15 respectively. The results indicate that the Fc variants display consistent binding enhancements regardless of the antibody context, and thus the Fc variants of the present invention are broadly applicable to antibodies and Fc fusions.

[211] Fc variants have been obtained that show differentially enhanced binding to FcγRIIIa over FcγRIIb. As discussed, optimal effector function may result from Fc variants wherein affinity for activating FcγRs is greater than affinity for the inhibitory FcγRIIb. AlphaScreen™ data directly comparing binding to FcγRIIIa and FcγRIIb for two Fc variants with this specificity profile are shown in Figures 16a and 16b. This concept can be defined quantitatively as the fold-enhancement or -reduction of the activating FγR (Table 61, column 3) divided by the fold-enhancement or -reduction of the inhibitory FcγR (Table 61, column 4), herein referred to as the FcγRIIIa-fold:FcγRIIb-fold ratio. This value provided in Column 5 in Table 61. Table 61 shows that Fc variants provide this specificity profile, with a FcγRIIIa-fold:FcγRIIb-fold ratio as high as 86:1.

[212] Some of the most promising Fc variants of the present invention for enhancing effector function have both substantial increases in affinity for FcγRIIIa and favorable FcγRIIIa-fold:FcγRIIb-fold ratios. These include, for example, S239D/I332E (FcγRIIIa-fold = 56, FcγRIIIa-fold:FcγRIIb-fold = 3), S239D/A330Y/I332E (FcγRIIIa-fold = 130), S239D/A330L/I332E (FcγRIIIa-fold = 139, FcγRIIIa-fold:FcγRIIb-fold = 18), and S239D/S298A/I332E (FcγRIIIa-fold = 295, FcγRIIIa-fold:FcγRIIb-fold = 48). Figure 17 shows AlphaScreen™ binding data for these and other Fc variants to human V158 FcγRIIIa.

[213] Because there are a number of FcγRs that contribute to effector function, it may be worthwhile to additionally screen Fc variants against other receptors. Figure 18 shows AlphaScreen™ data for binding of select Fc variants to human R131 FcγRIIa. As can be seen, those aforementioned variants

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with favorable binding enhancements and specificity profiles also show enhanced binding to this activating receptor. The use of FcγRIIIa, FcγRIIb, and FcγRIIc for screening is not meant to constrain experimental testing to these particular FcγRs; other FcγRs are contemplated for screening, including but not limited to the myriad isoforms and allotypes of FcγRI, FcγRII, and FcγRIII from humans, mice, rats, monkeys, and the like, as previously described.

[214] Taken together, the FcγR binding data provided in Figures 11 – 18 and Table 61 indicate that a number of substitions at positions 234, 235, 239, 240, 243, 264, 266, 325, 328, 330, and 332 are promising candidates for improving the effector function of antibodies and Fc fusions. Because combinations of some of these substitutions have typically resulted in additive or synergistic binding improvements, it is anticipated that as yet unexplored combinations of the Fc variants provided in Table 61 will also provide favorable results. Thus all combinations of the Fc variants in Table 61 are contemplated. Likewise, combinations of any of the Fc variants in Table 61 with other discovered or undiscovered Fc variants may also provide favorable properties, and these combinations are also contemplated. Furthermore, it is anticipated from these results that other substitutions at positions 234, 235, 239, 240, 243, 264, 266, 325, 328, 330, and 332 may also provide favorable binding enhancements and specificities, and thus substitutions at these positions other than those presented in Table 61 are contemplated.

[215] Example 4: Reduced binding to FcγRs

As discussed, although there is a need for greater effector function, for some antibody therapeutics, reduced or eliminated effector function may be desired. Several Fc variants in Table 61 substantially reduce or ablate FcγR binding, and thus may find use in antibodies and Fc fusions wherein effector function is undesirable. AlphaScreen™ binding data for some examples of such variants are shown in Figures 19a and 19b. These Fc variants, as well as their use in combination, may find use for eliminating effector function when desired, for example in antibodies and Fc fusions whose mechanism of action involves blocking or antagonism but not killing of the cells bearing target antigen.

[216] Example 5: Aglycosylated Fc variants

As discussed, one goal of the current experiments was to obtain optimized aglycosylated Fc variants. Several Fc variants provide significant progress towards this goal. Because it is the site of glycosylation, substitution at N297 results in an aglycosylated Fc. Whereas all other Fc variants that comprise a substitution at N297 completely ablate FcγR binding, N297D/I332E has significant binding affinity for FcγRIIIa, shown in Table 61 and illustrated in Figure 20. The exact reason for this result is uncertain in the absence of a high-resolution structure for this variant, although the computational screening predictions suggest that it is potentially due to a combination of new favorable Fc/FcγR interactions and favorable electrostatic properties. Indeed other electrostatic substitutions are

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envisioned for further optimization of aglycosylated Fc. Table 61 shows that other aglycosylated Fc variants such as S239D/N297D/I332E and N297D/A330Y/I332E provide binding enhancements that bring affinity for FcγRIIIa within 0.28- and 0.43-fold respectively of glycosylated WT alemtuzumab. Combinations of these variants with other Fc variants that enhance FcγR binding are contemplated, with the goal of obtaining aglycosylated Fc variants that bind one or more FcγRs with affinity that is approximately the same as or even better than glycosylated parent Fc. An additional set of promising Fc variants provide stability and solubility enhancements in the absence of carbohydrate. Fc variants that comprise substitutions at positions 241, 243, 262, and 264, positions that do not mediate FγR binding but do determine the interface between the carbohydrate and Fc, ablate FγR binding, presumably because they perturb the conformation of the carbohydrate. In deglycosylated form, however, Fc variants F241E/F243R/V262E/V264R, F241E/F243Q/V262T/V264E, F241R/F243Q/V262T/V264R, and F241E/F243Y/V262T/V264R show stronger binding to FcγRIIIa than in glycosylated form, as shown by the AlphaScreen™ data in Figure 21. This result indicates that these are key positions for optimization of the structure, stability, solubility, and function of aglycosylated Fc. Together these results suggests that protein engineering can be used to restore the favorable functional and solution properties of antibodies and Fc fusions in the absence of carbohydrate, and pave the way for aglycosylated antibodies and Fc fusions with favorable solution properties and full functionality that comprise substitutions at these and other Fc positions.

[217] Example 6. Affinity of Fc variants for polymorphic forms of FcγRIIIa

As discussed above, an important parameter of Fc-mediated effector function is the affinity of Fc for both V158 and F158 polymorphic forms of FcγRIIIa. AlphaScreen™ data comparing binding of select variants to the two receptor allotypes are shown in Figure 22a (V158 FcγRIIIa) and Figure 22b (F158 FcγRIIIa). As can be seen, all variants improve binding to both FcγRIIIa allotypes. These data indicate that those Fc variants of the present invention with enhanced effector function will be broadly applicable to the entire patient population, and that enhancement to clinical efficacy will potentially be greatest for the low responsive patient population who need it most.

[218] The FcγR binding affinities of these Fc variants were further investigated using Surface Plasmon Resonance (SPR) (Biacore, Uppsala, Sweden). SPR is a sensitive and extremely quantitative method that allows for the measurement of binding affinities of protein-protein interactions, and has been used to effectively measure Fc/FcγR binding (Radaev *et al.*, 2001, *J Biol Chem* 276:16478-16483). SPR thus provides an excellent complementary binding assay to the AlphaScreen™ assay. His-tagged V158 FcγRIIIa was immobilized to an SPR chip, and WT and Fc variant alemtuzumab antibodies were flowed over the chip at a range of concentrations. Binding constants were obtained from fitting the data using standard curve-fitting methods. Table 62 presents dissociation constants (Kd) for binding of select Fc variants to V158 FcγRIIIa and F158 FcγRIIIa obtained using SPR, and compares these with IC50s obtained from the AlphaScreen™ assay. By

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dividing the Kd and IC50 for each variant by that of WT alemtuzumab, the fold-improvements over WT (Fold) are obtained.

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Table 62

	SPR V158 FcγRIIIa Kd (nM)	Fold	SPR F158 FcγRIIIa Kd (nM)	Fold	AlphaScreen™ V158 FcγRIIIa IC50 (nM)	Fold	AlphaScreen™ F158 FcγRIIIa IC50 (nM)	Fold
WT	68		730		6.4		17.2	
V264I	64	1.1	550	1.3	4.5	1.4	11.5	1.5
I332E	31	2.2	72	10.1	1.0	6.4	2.5	6.9
V264I/I332E	17	4.0	52	14.0	0.5	12.8	1.1	15.6
S298A	52	1.3	285	2.6	2.9	2.2	12.0	1.4
S298A/E333A/ K334A	39	1.7	156	4.7	2.5	2.6	7.5	2.3

[219] The SPR data corroborate the improvements to FcγRIIIa affinity observed by AlphaScreen™ assay. Table 62 further indicates the superiority of V264I/I332E and I332E over S298A and S298A/E333A/K334A; whereas S298A/E333A/K334A improves Fc binding to V158 and F158 FcγRIIIa by 1.7-fold and 4.7-fold respectively, I332E shows binding enhancements of 2.2-fold and 10.1-fold respectively, and V264I/I332E shows binding enhancements of 4.0-fold and 14-fold respectively. Also worth noting is that the affinity of V264I/I332E for F158 FcγRIIIa (52 nM) is better than that of WT for the V158 allotype (68 nM), suggesting that this Fc variant, as well as those with even greater improvements in binding, may enable the clinical efficacy of antibodies for the low responsive patient population to achieve that currently possible for high responders. The correlation between the SPR and AlphaScreen™ binding measurements are shown in Figures 23a – 23d. Figures 23a and 23b show the Kd - IC50 correlations for binding to V158 FcγRIIIa and F158 FcγRIIIa respectively, and Figures 23c and 23d show the fold-improvement correlations for binding to V158 FcγRIIIa and F158 FcγRIIIa respectively. The good fits of these data to straight lines (r^2 = 0.9, r^2 = 0.84, r^2 = 0.98, and r^2 = 0.90) support the accuracy the AlphaScreen™ measurements, and validate its use for determining the relative Fc□R binding affinities of Fc variants.

[220] Example 7. ADCC of Fc variants

In order to determine the effect on effector function, cell-based ADCC assays were performed on select Fc variants. ADCC was measured using the DELFIA® EuTDA-based cytotoxicity assay (Perkin Elmer, MA) with purified human peripheral blood monocytes (PBMCs) as effector cells. Target cells were loaded with BATDA at $1x10^6$ cells/ml, washed 4 times and seeded into 96-well plate at 10,000 cells/well. The target cells were then opsonized using Fc variant or WT antibodies at the indicated final concentration. Human PBMCs were added at the indicated fold-excess of target cells and the plate was incubated at 37°C for 4 hrs. The co-cultured cells were centrifuged at 500xg, supernatants were transferred to a separate plate and incubated with Eu solution, and relative fluorescence units were measured using a Packard Fusion™ reader (Packard Biosciences, IL). Samples were run in triplicate to provide error estimates (n=3, +/- S.D.). PBMCs were allotyped for the V158 or F158 FcγRIIIa allotype using PCR.

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[221] ADCC assays were run on Fc variant and WT alemtuzumab using DoHH-2 lymphoma target cells. Figure 24a is a bar graph showing the ADCC of these proteins at 10 ng/ml antibody. Results show that alemtuzumab Fc variants I332E, V264I, and I332E/V264I have substantially enhanced ADCC compared to WT alemtuzumab, with the relative ADCC enhancements proportional to their binding improvements to FcγRIIIa as indicated by AlphaScreen™ assay and SPR. The dose dependence of ADCC on antibody concentration is shown in Figure 24b. These data were normalized to the minimum and maximum fluorescence signal provided by the baselines at low and high concentrations of antibody respectively. The data were fit to a sigmoidal dose-response model using nonlinear regression, represented by the curve in the figure. The fits enable determination of the effective concentration 50% (EC50) (i.e. the concentration required for 50% effectiveness), which provides the relative enhancements to ADCC for each Fc variant. The EC50s for these binding data are analogous to the IC50s obtained from the AlphaScreen™ competition data, and derivation of these values is thus analogous to that described in Example 2 and Figure 11. In Figure 24b, the log(EC50)s, obtained from the fits to the data, for WT, V264I/I332E, and S239D/I332E alemtuzumab are 0.99, 0.60, and 0.49 respectively, and therefore their respective EC50s are 9.9, 4.0, and 3.0. Thus V264I/I332E and S239E/I332E provide a 2.5-fold and 3.3-fold enhancement respectively in ADCC over WT alemtuzumab using PBMCs expressing heterozygous V158/F158 FcγRIIIa. These data are summarized in Table 63 below.

Table 63

	log(EC50)	EC50 (ng/ml)	Fold Improvement Over WT
WT	0.99	9.9	
V264I/I332E	0.60	4.0	2.5
S239D/I332E	0.49	3.0	3.3

[222] In order to determine whether these ADCC enhancements are broadly applicable to antibodies, select Fc variants were evaluated in the context of rituximab and trastuzumab. ADCC assays were run on V264I/I332E, WT, and S298A/D333A/K334A rituximab using WIL2-S lymphoma target cells. Figure 25a presents a bar graph showing the ADCC of these proteins at 1 ng/ml antibody. Results indicate that V264I/I332E rituximab provides substantially enhanced ADCC relative to WT rituximab, as well as superior ADCC to S298A/D333A/K334A, consistent with the FcγRIIIa binding improvements observed by AlphaScreen™ assay and SPR. Figure 25b shows the dose dependence of ADCC on antibody concentration. The EC50s obtained from the fits of these data and the relative fold-improvements in ADCC are provided in Table 64 below. As can be seen V264I/I332E rituximab provides an 11.3-fold enhancement in EC50 over WT for PBMCs expressing homozygous F158/F158 FcγRIIIa. The greater improvements observed for rituximab versus alemtuzumab are

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likely due to the use of homozygous F158/F158 FcγRIIIa rather than heterozygous V158/F158 FcγRIIIa PBMCs, as well as potentially the use of different antibodies and target cell lines.

Table 64

	log(EC50)	EC50 (ng/ml)	Fold Improvement Over WT
WT	0.23	1.7	
S298A/E333A/K334A	-0.44	0.37	4.6
V264I/I332E	-0.83	0.15	11.3

[223] ADCC assays were run on Fc variant and WT trastuzumab using two breast carcinoma target cell lines BT474 and Sk-Br-3. Figure 26a shows a bar graph illustrating ADCC at 1 ng/ml antibody. Results indicate that V264I and V264I/I332E trastuzumab provide substantially enhanced ADCC compared to WT trastuzumab, with the relative ADCC enhancements proportional to their binding improvements to FcγRIIIa as indicated by AlphaScreen™ assay and SPR. Figure 26b shows the dose dependence of ADCC on antibody concentration. The EC50s obtained from the fits of these data and the relative fold-improvements in ADCC are provided in Table 65 below. Significant ADCC improvements are observed for I332E trastuzumab when combined with A330L and A330Y.

Table 65

	log(EC50)	EC50 (ng/ml)	Fold Improvement Over WT
WT	1.1	11.5	
I332E	0.34	2.2	5.2
A330Y/I332E	-0.04	0.9	12.8
A330L/I332E	0.04	1.1	10.5

[224] Figure 26c shows another set of dose response ADCC data at variable antibody concentrations for trastuzumab variants. The EC50s obtained from the fits of these data and the relative fold-improvements in ADCC are provided in Table 66 below. Results show that trastuzumab Fc variants S239D/I332E, S239D/S298A/I332E, S239D/A330Y/I332E, and S239D/A330L/I332E/ provide substantial ADCC enhancements relative to WT trastuzumab and S298A/E333A/K334A; consistent with the FcγR binding data observed by the AlphaScreen™ assay and SPR. S239D/A330L/I332E trastuzumab shows the largest increase in effector function observed thus far, providing an approximate 50-fold enhancement in EC50 over WT for PBMCs expressing homozygous F158/F158 FcγRIIIa.

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Table 66

	log(EC50)	EC50 (ng/ml)	Fold Improvement Over WT
WT	0.45	2.83	
S298A/E333A/K334A	-0.17	0.67	4.2
S239D/I332E	-0.18	0.66	4.3
S239D/A330Y/I332E	-0.29	0.51	5.5
S239D/S298A/I332E	-0.52	0.30	9.4
S239D/A330L/I332E	-1.22	0.06	47.2

[225] Example 8. Complement binding and activation by Fc variants

Complement protein C1q binds to a site on Fc that is proximal to the FcγR binding site, and therefore it was prudent to determine whether the Fc variants have maintained their capacity to recruit and activate complement. The AlphaScreen™ assay was used to measure binding of select Fc variants to the complement protein C1q. The assay was carried out with biotinylated WT alemtuzumab antibody attached to streptavidin donor beads as described in Example 2, and using C1q coupled directly to acceptor beads. Binding data of select Fc variants shown in Figure 27a indicate that C1q binding is uncompromised. Cell-based CDC assays were also performed on select Fc variants to investigate whether Fc variants maintain the capacity to activate complement. Amar Blue was used to monitor lysis of Fc variant and WT rituximab-opsonized WIL2-S lymphoma cells by human serum complement (Quidel, San Diego, CA). The results shown in Figure 27b for select Fc variants indicate that CDC is uncompromised.

[226] Example 9. Protein A binding by Fc variants

As discussed, bacterial protein A binds to the Fc region between the C□2 and C□3 domains, and is frequently employed for antibody purification. The AlphaScreen™ assay was used to measure binding of select Fc variants to the protein A using biotinylated WT alemtuzumab antibody attached to streptavidin donor beads as described in Example 2, and using protein A coupled directly to acceptor beads. The binding data shown in Figure 28 for select Fc variants indicate that the capacity of the Fc variants to bind protein A is uncompromised. These results suggest that affinity of the Fc variants for other Fc ligands that bind the same site on Fc as protein A, such as the neonatal Fc receptor FcRn and protein G, are also unaffected.

[227] Example 10. Capacity of Fc variants to bind mouse Fc□Rs

Optimization of Fc to nonhuman Fc□Rs may be useful for experimentally testing Fc variants in animal models. For example, when tested in mice (for example nude mice, SCID mice, xenograft mice, and/or transgenic mice), antibodies and Fc fusions that comprise Fc variants that are optimized for

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one or more mouse Fc□Rs may provide valuable information with regard to efficacy, mechanism of action, and the like. In order to evaluate whether the Fc variants of the present invention may be useful in such experiments, affinity of select Fc variants for mouse FcγRIII was measured using the AlphaScreen™ assay. The AlphaScreen™ assay was carried out using biotinylated WT alemtuzumab attached to streptavidin donor beads as described in Example 2, and GST-tagged mouse FcγRIII bound to glutathione chelate acceptor beads, expressed and purified as described in Example 2. These binding data are shown in Figure 29. Results show that some Fc variants that enhance binding to human FcγRIIIa also enhance binding to mouse FcγRIII. This result indicates that the Fc variants of the present invention, or other Fc variants that are optimized for nonhuman FcγRs, may find use in experiments that use animal models.

[228] Example 11. Validation of Fc variants expressed in CHO cells

Whereas the Fc variants of the present invention were expressed in 293T cells for screening purposes, large scale production of antibodies is typically carried out by expression in Chinese Hamster Ovary (CHO) cell lines. In order to evaluate the properties of CHO-expressed Fc variants, select Fc variants and WT alemtuzumab were expressed in CHO cells and purified as described in Example 2. Figure 30 shows AlphaScreen™ data comparing binding of CHO- and 293T- expressed Fc variant and WT alemtuzumab to human V158 FcγRIIIa. The results indicate that the Fc variants of the present invention show comparable FcγR binding enhancements whether expressed in 293T or CHO.

[229] Example 12. Therapeutic application of Fc variants

A number of Fc variants described in the present invention have significant potential for improving the therapeutic efficacy of anticancer antibodies. For illustration purposes, a number of Fc variants of the present invention have been incorporated into the sequence of the antibody rituximab. The WT rituximab light chain and heavy chain, described in US 5,736,137, are provided in Figures 31a and 32b. The improved anti-CD20 antibody sequences are provided in Figure 31c. The improved anti-CD20 antibody sequences comprise at least non-WT amino acid selected from the group consisting of X_1, X_2, X_3, X_4, X_5, and X_6. These improved anti-CD20 antibody sequences may also comprise a substitution Z_1. The use of rituximab here is solely an example, and is not meant to constrain application of the Fc variants to this antibody or any other particular antibody or Fc fusion.

[230] All references are herein expressly incorporated by reference.

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[231] Whereas particular embodiments of the invention have been described above for purposes of illustration, it will be appreciated by those skilled in the art that numerous variations of the details may be made without departing from the invention as described in the appended claims.

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CLAIMS

We claim:

1. An antibody comprising an Fc variant portion comprising at least one amino acid modification in the Fc region of said parent Fc polypeptide, wherein said Fc variant modulates binding to an FcγR as compared to the parent Fc polypeptide.

2. An antibody according to claim 1 wherein said modulation is an increase in affinity of said antibody to said FcγR.

3. An antibody according to claim 1 wherein said Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 267, 269, 296, 297, 298, 299, 313, 325, 326, 327, 328, 329, 330, 332, 333, and 334.

4. An antibody according to claim 1, wherein said Fc variant comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 240, 244, 245, 247, 262, 263, 266, 299, 313, 325, 328, and 332.

5. An antibody according to claim 1, wherein said Fc variant comprises a substitution at position 332.

6. An antibody according to claim 1, wherein at least one substitution is at a position selected from the group consisting of: 239, 264, 297, and 330, with the proviso that if said sequence is substantially human, said substitution is not S239A, V264A, N297A, N297Q, A330D, A330Q, A330K, or A330S.

7. An antibody according to claim 1, wherein said Fc variant comprises at least one substitution selected from the group consisting of 234D, 234E, 234N, 234Q, 234T, 234H, 234Y, 234I, 234V, 234F, 235D, 235S, 235N, 235Q, 235T, 235H, 235Y, 235I, 235V, 235F, 239D, 239E, 239N, 239Q, 239F, 239T, 239H, 239Y, 240I, 240A, 240T, 240M, 241W, 241L, 241Y, 241E, 241R, 243W, 243L 243Y, 243R, 243Q, 244H, 245A, 247V, 247G, 262I, 262A, 262T, 262E, 263I, 263A, 263T, 263M, 264L, 264I, 264W, 264T, 264R, 264F, 264M, 264Y, 264E, 265G, 265N, 265Q, 265Y, 265F, 265V, 265I, 265L, 265H, 265T, 266I, 266A, 266T, 266M, 267Q, 267L, 269H, 269Y, 269F, 269R, 296E, 296Q, 296D, 296N, 296S, 296T, 296L, 296I, 296H,

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297S, 297D, 297E, 298H, 299I, 299L, 299A, 299S, 299V, 299H, 299F, 299E, 313F, 325Q, 325L, 325I, 325D, 325E, 325A, 325T, 325V, 325H, 327N, 327L, 328M, 328D, 328E, 328N, 328Q, 328F, 328I, 328V, 328T, 328H, 328A, 329F, 330L, 330Y, 330V, 330I, 330F, 330R, 330H, 332D, 332E, 332N, 332Q, 332T, 332H, 332Y, and 332A.

8. An antibody according to claim 1, wherein said Fc variant is selected from the group consisting of 264L, 264I, 241W, 241L, 243W, 243L, 241L/243L/262I/264I, 241W/243W, 241W/243W/262A/264A, 241L/262I, 243L/264I, 243L/262I/264W, 241Y/243Y/262T/264T, 241E/243R/262E/264R, 241E/243Q/262T/264E, 241R/243Q/262T/264R, 241E/243Y/262T/264R, 328M, 328E, 328F, 332E, 328M/332E, 244H, 245A, 247V, 313F, 244H/245A/247V, 247G, 264I/332E, 241E/243R/262E/264R/332E, 241E/243Q/262T/264E/332E, 241R/243Q/262T/264R/332E, 241E/243Y/262T/264R/332E, 298A/332E, 239EI332E, 239Q/332E, 239E, 265G, 265N, 239E/265G, 239E/265N, 239E/265Q, 296E, 296Q, 299I, 327N, 267Q/327S, 267L/327S , 327L, 329F, 330L, 330Y, 332D, 297S, 297D, 297S/332E, 297D/332E, 297E/332E, 265Y/297D/332E, 265Y/297D/299L/332E, 265F/297E/332E, 328I/332E, 328Q/332E, 332N, 332Q, 264T, 264F, 240I, 263I, 266I, 299A, 299S, 299V, 325Q, 325L, 325I, 239D, 239N, 239F, 239D/332D, 239D/332E, 239D/332N, 239D/332Q, 239E/332D, 239E/332N, 239E/332Q, 239N/332D, 239N/332E, 239N/332N, 239NI332Q, 239Q/332D, 239Q/332N, 239Q/332Q, 296D, 296N, 241Y/243Y/262T/264T/297D/332E, 330Y/332E, 264I/330Y/332E, 330L/332E, 264I/330L/332E, 234D, 234E, 234N, 234Q, 234T, 234H, 234Y, 234I, 234V, 234F, 235D, 235S, 235N, 235Q, 235T, 235H, 235Y, 235I, 235V, 235F, 239T, 239H, 239Y, 240A, 240T, 240M, 263A, 263T, 263M, 264M, 264Y, 266A, 266T, 266M, 269H, 269Y, 269F, 269R, 296S, 296T, 296L, 296I, 298H, 299H, 330V, 330I, 330F, 330R, 330H, 325D, 325E, 325A, 325T, 325V, 325H, 328D/332E, 328E/332E, 328N/332E, 328Q/332E, 328V/332E, 328T/332E, 328H/332E, 328I/332E, 328A, 332T, 332H, 332Y, 332A, 239E/264I/332E, 239Q/264I/332E, 239E/264I/330Y/332E, 239E/264I/298A/330Y/332E, 239D/297D/332E, 239E/297D/332E, 239D/265V/297D/332E, 239D/265I/297D/332E, 239D/265L/297D/332E, 239D/265F/297D/332E, 239D/265Y/297D/332E, 239D/265H/297D/332E, 239D/265T/297D/332E, 264E/297D/332E, 296D/297D/332E, 296E/297D/332E, 296N/297D/332E, 296Q/297D/332E, 296H/297D/332E, 296T/297D/332E, 297D/299V/332E, 297D/299I/332E, 297D/299L/332E, 297D/299F/332E, 297D/299H/332E, 297D/299E/332E, 297D/330Y/332E, 297D/298A/330YI332E, 239D/330Y/332E, 239N/330Y/332E, 239D/330L/332E, 239N/330L/332E, 264I/298A/332E, 239D/298A/332E, 239N/298A/332E, 239D/264I/332E, 239D/264I/298A/332E, and 239D/264I/330L/332E.

9. An antibody according to claim 7, wherein said Fc variant portion further comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 256, 270, 290, 298, 312, 322, 326, 329, 331, 333, 334, and 339.

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10. An antibody according to claim 1, wherein said FcγR is FcγRIIIa.

11. An antibody according to claim 10, wherein said FcγRIIIa is a V158 or F158 allotype of FcγRIIIa.

12. An Fc variant according to claim 2, wherein said parent Fc polypeptide is substantially human, and said affinity is approximately 5-fold greater than that of said parent Fc polypeptide.

13. An antibody according to claim 12, wherein said parent Fc polypeptide is substantially human, and said affinity is between about 5-fold and about 300-fold greater than that of said parent Fc polypeptide.

14. An antibody according to claim 12, wherein said Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting 234, 235, 239, 240, 243, 264, 266, 328, 330, 332, and 325.

15. An antibody according to claim 14, wherein said Fc variant comprises at least one substitution selected from the group consisting of 234E, 234Y, 234I, 235D, 235S, 235Y, 235I, 239D, 239E, 239N, 239Q, 239T, 240I, 240M, 243L, 264I, 264T, 264Y, 266I, 328M, 328I, 328Q, 328D, 328V, 328T, 330Y, 330L, 330I, 332D, 332E, 332N, 332Q, and 325T.

16. An antibody according to claim 15, wherein said Fc variant is selected from the group consisting of 264I, 243L/264I, 328M, 332E, 328M/332E, 264I/332E, 298A/332E, 239E/332E, 239Q/332E, 239E, 330Y, 332D, 328I/332E, 328Q/332E, 264T, 240I, 266I, 239D, 239D/332D, 239D/332E, 239D/332N, 239D/332Q, 239E/332D, 239E/332N, 239E/332Q, 239N/332D, 239N/332E, 239Q/332D, 330Y/332E, 264I/330Y/332E, 330L/332E, 264I/330L/332E, 234E, 234Y, 234I, 235D, 235S, 235Y, 235I, 239T, 240M, 264Y, 330I, 325T, 328D/332E, 328V/332E, 328T/332E, 328I/332E, 239E/264I/332E, 239Q/264I/332E, 239E/264I/330Y/332E, 239D/330Y/332E, 239N/330Y/332E, 239D/330L/332E, 239N/330L/332E, 264I/298A/332E, 239D/298A/332E, 239N/298A/332E, 239D/264I/332E, 239D/264I/298A/332E, and 239D/264I/330L/332E.

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17. An antibody according to claim 15, wherein said Fc variant further comprises at least one substitution at a position selected from the group consisting of 256, 270, 290, 298, 312, 322, 326, 329, 331, 333, 334, and 339.

18. An antibody according to claim 1 or 2, wherein said parent Fc polypeptide is substantially human, substantially mouse, substantially rat, or substantially monkey.

19. An antibody according to claim 1 or 2, wherein binding to one or more Fc ligands is unaltered.

20. An antibody according to claim 19, wherein said Fc ligand is selected from the group consisting of C1q, FcRn, protein A, and protein G.

21. An antibody according to claim 1 or 2, wherein CDC is unaffected.

22. An antibody according to claim 1 or 2, wherein binding to one or more Fc ligands is altered.

23. An antibody according to claim 1, wherein said Fc variant has a FcγRIIIa-fold:FcγRIIb-fold ratio greater than 1.

24. An antibody according to claim 23, wherein said Fc variant has a FcγRIIIa-fold:FcγRIIb-fold ratio greater than approximately 11:1.

25. An antibody according to claim 24, wherein said Fc variant has a FcγRIIIa-fold:FcγRIIb-fold ratio between approximately 11:1 and approximately 86:1.

26. An antibody according to claim 23, wherein said Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 234, 235, 239, 240, 264, 296, 330, and I332.

27. An antibody according to claim 26, wherein said Fc variant comprises at least one substitution selected from the group consisting of: 234Y, 234I, 235I, 239D, 239E, 239N, 239Q, 240A, 240M, 264I, 264Y, 296Q, 330L, 330Y, 330I, 332D, and 332E.

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28. An antibody according to claim 27, wherein said Fc variant is selected from the group consisting of 332E, 264I/332E, 239E/332E, 239Q/332E, 296Q, 330L, 330Y, 332D, 239D, 239D/332E, 330Y/332E, 264I/330Y/332E, 330L/332E, 264I/330L/332E, 234Y, 234I, 235I, 240A, 240M, 264Y, 330I, 239D/330L/332E, 239D/298A/332E, 239N/298A/332E, 239D/264I/332E, 239D/264I/298A/332E, and 239D/264I/330L/332E.

29. An antibody according to claim 26, wherein said Fc variant further comprises one or more substitutions at a position selected from the group consisting of 256, 270, 290, 298, 312, 322, 326, 329, 331, 333, 334, and 339.

30. An antibody according to claim 1, wherein said Fc variant binds to at least one FcγR with reduced affinity relative to the parent Fc polypeptide.

31. An antibody according to claim 30, wherein said FcγR is FcγRIIIa.

32. An antibody according to claim 31, wherein said Fc variant comprises at least one substitution selected from the group consisting of 234D, 234N, 234Q, 234T, 234H, 234V, 234F, 235N, 235Q, 235T, 235H, 235V, 235F, 239E, 239N, 239Q, 239F, 239H, 239Y, 240A, 240T, 241W, 241L, 241Y, 241E, 241R, 243W, 243L 243Y, 243R, 243Q, 244H, 245A, 247V, 247G, 262I, 262A, 262T, 262E, 263I, 263A, 263T, 263M, 264L, 264I, 264W, 264T, 264R, 264F, 264M, 264E, 265G, 265N, 265Q, 265Y, 265F, 265V, 265I, 265L, 265H, 265T, 266A, 266T, 266M, 267Q, 267L, 269H, 269Y, 269F, 269R, 296E, 296Q, 296D, 296N, 296S, 296T, 296L, 296I, 296H, 297S, 297D, 297E, 298H, 299I, 299L, 299A, 299S, 299V, 299H, 299F, 299E, 313F, 325Q, 325L, 325I, 325D, 325E, 325A, 325V, 325H, 327N, 327L, 328M, 328E, 328N, 328Q, 328F, 328H, 328A, 329F, 330L, 330V, 330F, 330R, 330H, 332N, 332Q, 332T, 332H, 332Y, and 332A

33. An antibody according to claim 31, wherein said Fc variant is selected from the group consisting of 264L, 241W, 241L, 243W, 243L, 241L/243L/262I/264I, 241W/243W, 241W/243W/262A/264A, 241L/262I, 243L/262I/264W, 241Y/243Y/262T/264T, 241E/243R/262E/264R, 241E/243Q/262T/264E, 241R/243Q/262T/264R, 241E/243Y/262T/264R, 328M, 328E, 328F, 244H, 245A, 247V, 313F, 244H/245A/247V, 247G, 241E/243R/262E/264R/332E, 241E/243Y/262T/264R/332E, 265G, 265N, 239E/265G, 239E/265N, 239E/265Q, 296E, 296Q, 299I, 327N, 267Q/327S, 267L/327S , 327L, 329F, 330L, 297S, 297D, 297S/332E, 332N, 332Q, 264F, 263I, 299A, 299S, 299V, 325Q, 325L, 325I, 239N, 239F, 239N/332N, 239N/332Q, 239Q/332N, 239Q/332Q, 296D, 296N, 234D, 234N, 234Q, 234T, 234H, 234V, 234F, 235N, 235Q, 235T, 235H, 235V, 235F, 239H, 239Y,

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240A, 263T, 263M, 264M, 266A, 266T, 266M, 269H, 269Y, 269F, 269R, 296S, 296T, 296L, 296I, 298H, 299H, 330V, 330F, 330R, 330H, 325D, 325E, 325A, 325V, 325H, 328E/332E, 328N/332E, 328Q/332E, 328H/332E, 328A, 332T, 332H, 332Y, and 332A.

34. An antibody comprising a variant of a parent Fc polypeptide comprising at least one amino acid modification in the Fc region of said parent Fc polypeptide, wherein said Fc variant modulates effector function as compared to the parent Fc polypeptide.

35. An antibody according to claim 34, wherein said effector function is ADCC.

36. An antibody according to claim 35, wherein said Fc variant improves ADCC as compared to said parent Fc polypeptide.

37. An antibody according to claim 36, wherein said ADCC improvement is approximately 5-fold greater than that of said parent Fc polypeptide.

38. An antibody according to claim 37, wherein said ADCC improvement is between approximately 5-fold and 50-fold greater than that of said parent Fc polypeptide.

39. An antibody according to claim 37, wherein said Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 234, 235, 239, 240, 243, 264, 266, 328, 330, 332, and 325.

40. An antibody according to claim 39, wherein said Fc variant comprises at least one substitution selected from the group consisting of 234E, 234Y, 234I, 235D, 235S, 235Y, 235I, 239D, 239E, 239N, 239Q, 239T, 240I, 240M, 243L, 264I, 264T, 264Y, 266I, 328M, 328I, 328Q, 328D, 328V, 328T, 330Y, 330L, 330I, 332D, 332E, 332N, 332Q, and 325T.

41. An antibody according to claim 39, wherein said Fc variant is selected from the group consisting of 264I, 243L/264I, 328M, 332E, 328M/332E, 264I/332E, 298A/332E, 239E/332E, 239Q/332E, 239E, 330Y, 332D, 328I/332E, 328Q/332E, 264T, 240I, 266I, 239D, 239D/332D, 239D/332E, 239D/332N, 239D/332Q, 239E/332D, 239E/332N, 239E/332Q, 239N/332D, 239N/332E, 239Q/332D, 330Y/332E, 264I/330Y/332E, 330L/332E, 264I/330L/332E, 234E, 234Y, 234I, 235D, 235S, 235Y, 235I, 239T, 240M, 264Y, 330I, 325T, 328D/332E, 328V/332E, 328T/332E, 328I/332E, 239E/264I/332E, 239Q/264I/332E, 239E/264I/330Y/332E,

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239D/330Y/332E, 239N/330Y/332E, 239D/330L/332E, 239N/330L/332E, 264I/298A/332E, 239D/298A/332E, 239N/298A/332E, 239D/264I/332E, 239D/264I/298A/332E, and 239D/264I/330L/332E.

42. An antibody according to claim 39, wherein said Fc variant further comprises at least one substitution at a position selected from the group consisting of 256, 270, 290, 298, 312, 322, 326, 329, 331, 333, 334, and 339.

43. An antibody according to claim 39, wherein said parent Fc polypeptide is substantially human, substantially mouse, substantially rat, or substantially monkey.

44. An antibody according to claim 35, wherein said Fc variant reduces ADCC as compared to said parent Fc polypeptide.

45. An antibody according to claim 44, wherein said Fc variant comprises at least one substitution selected from the group consisting of 234D, 234N, 234Q, 234T, 234H, 234V, 234F, 235N, 235Q, 235T, 235H, 235V, 235F, 239E, 239N, 239Q, 239F, 239H, 239Y, 240A, 240T, 241W, 241L, 241Y, 241E, 241R, 243W, 243L 243Y, 243R, 243Q, 244H, 245A, 247V, 247G, 262I, 262A, 262T, 262E, 263I, 263A, 263T, 263M, 264L, 264I, 264W, 264T, 264R, 264F, 264M, 264E, 265G, 265N, 265Q, 265Y, 265F, 265V, 265I, 265L, 265H, 265T, 266A, 266T, 266M, 267Q, 267L, 269H, 269Y, 269F, 269R, 296E, 296Q, 296D, 296N, 296S, 296T, 296L, 296I, 296H, 297S, 297D, 297E, 298H, 299I, 299L, 299A, 299S, 299V, 299H, 299F, 299E, 313F, 325Q, 325L, 325I, 325D, 325E, 325A, 325V, 325H, 327N, 327L, 328M, 328E, 328N, 328Q, 328F, 328H, 328A, 329F, 330L, 330V, 330F, 330R, 330H, 332N, 332Q, 332T, 332H, 332Y, and 332A.

46. An antibody according to claim 44, wherein said Fc variant is selected from the group consisting of 264L, 241W, 241L, 243W, 243L, 241L/243L/262I/264I, 241W/243W, 241W/243W/262A/264A, 241L/262I, 243L/262I/264W, 241Y/243Y/262T/264T, 241E/243R/262E/264R, 241E/243Q/262T/264E, 241R/243Q/262T/264R, 241E/243Y/262T/264R, 328M, 328E, 328F, 244H, 245A, 247V, 313F, 244H/245A/247V, 247G, 241E/243R/262E/264R/332E, 241E/243Y/262T/264R/332E, 265G, 265N, 239E/265G, 239E/265N, 239E/265Q, 296E, 296Q, 299I, 327N, 267Q/327S, 267L/327S , 327L, 329F, 330L, 297S, 297D, 297S/332E, 332N, 332Q, 264F, 263I, 299A, 299S, 299V, 325Q, 325L, 325I, 239N, 239F, 239N/332N, 239N/332Q, 239Q/332N, 239Q/332Q, 296D, 296N, 234D, 234N, 234Q, 234T, 234H, 234V, 234F, 235N, 235Q, 235T, 235H, 235V, 235F, 239H, 239Y, 240A, 263T, 263M, 264M, 266A, 266T, 266M, 269H, 269Y, 269F, 269R, 296S, 296T, 296L,

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296I, 298H, 299H, 330V, 330F, 330R, 330H, 325D, 325E, 325A, 325V, 325H, 328E/332E, 328N/332E, 328Q/332E, 328H/332E, 328A, 332T, 332H, 332Y, and 332A.

47. An antibody comprising an aglycosylated Fc variant of a parent Fc polypeptide comprising at least one amino acid modification in the Fc region of said parent Fc polypeptide, wherein said aglycosylated Fc variant has improved stability, solubility, or binding affinity to an Fc ligand relative to the aglycosylated form of said parent Fc polypeptide.

48. An antibody according to claim 47, wherein said aglycosylated Fc variant has improved binding affinity to an Fc ligand as compared to the aglycosylated form of the parent Fc polypeptide.

49. An antibody according to claim 48, wherein said Fc ligand is an FcγR.

50. An antibody according to claim 49, wherein said FcγR is FcγRIIIa.

51. An antibody according to claim 48, wherein said improved binding affinity is within 0.4-fold of the glycosylated form of the parent Fc polypeptide.

52. An antibody according to claim 47, wherein said Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence selected from the group consisting of: 239, 241, 243, 262, 264, 265, 296, 297, 330, and 332.

53. An antibody according to claim 52, wherein said Fc variant comprises one or more substitutions selected from the group consisting of 239D, 239E, 241Y, 243Y, 262T, 264T, 264E, 265Y, 265H, 296N, 297D, 330Y, and 332E.

54. An antibody according to claim 52, wherein said Fc variant is selected from the group consisting of 297D/332E, 241Y/243Y/262T/264T/297D/332E, 239D/297D/332E, 239E/297D/332E, 239D/265Y/297D/332E, 239D/265H/297D/332E, 264E/297D/332E, 296N/297D/332E, and 297D/330Y/332E.

55. An antibody according to claim 53, wherein said aglycosylated Fc variant portion comprises at least one substitution at a position corresponding to a position of the human sequence

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selected from the group consisting of: 256, 270, 290, 298, 312, 322, 326, 329, 331, 333, 334, and 339.

56. An antibody according to claim 1 comprising a Fc fusion comprising said Fc variant.

57. An antibody according to claim 1 or 56, wherein said antibody further comprises an engineered glycoform.

58. An antibody according to claim 57, wherein said engineered glycoform improves effector function.

59. A pharmaceutical composition comprising an antibody according to claim 1 and a pharmaceutically acceptable carrier.

60. A method of treating a mammal in need of said treatment, comprising administering an antibody of claim 1.

61. An antibody comprising an Fc variant, said Fc variant comprises at least one substitution selected from the group consisting of: 332D, 332E, 332N, or 332Q, wherein said positions corresponding to the human sequence position, wherein said antibody has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33, CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

62. An antibody comprising an Fc variant, said Fc variant comprises at least one substitution selected from the group consisting of 264I, 264T, or 264Y, wherein said positions corresponding to the human sequence position, wherein said antibody or Fc fusion has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33, CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

63. An antibody comprising an Fc variant, said Fc variant comprises at least one substitution selected from the group consisting of 239D, 239E, 239N, 239Q, or 239T, wherein said positions corresponding to the human sequence position, wherein said antibody or Fc fusion has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33,

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CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

64. An antibody comprising an Fc variant, said Fc variant comprises at least one substitution selected from the group consisting of 330Y, 330L, or 330I, wherein said positions corresponding to the human sequence position, wherein said antibody or Fc fusion has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33, CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

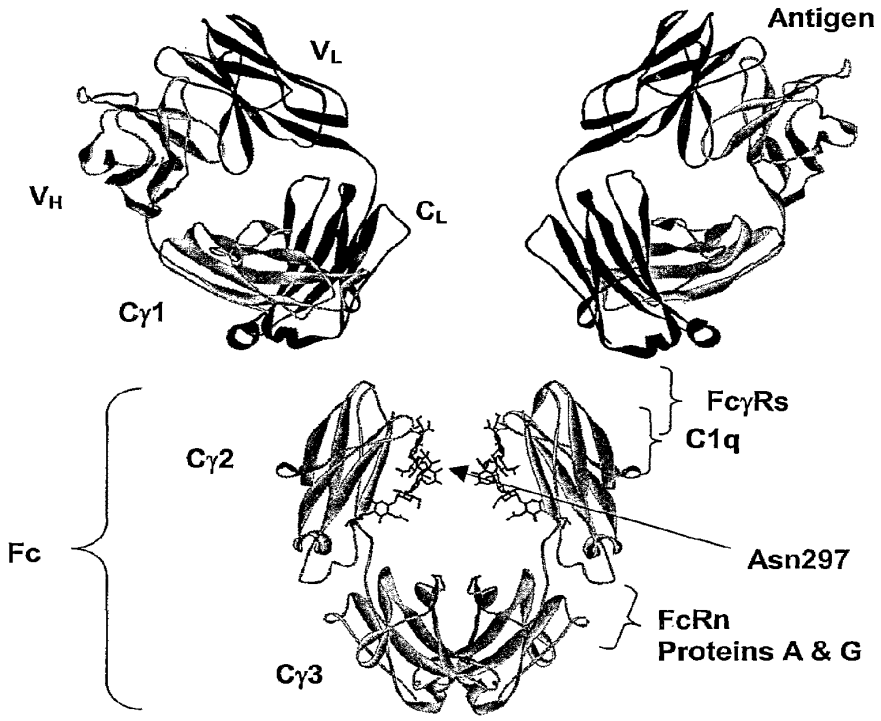
65. An antibody comprising an Fc variant, said Fc variant comprises at least one substitution selected from the group consisting of 240I or 240M, wherein said positions corresponding to the human sequence position, wherein said antibody or Fc fusion has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33, CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

66. An antibody comprising an Fc variant, said Fc variant comprises a substitution consisting of 297D, wherein said position corresponds to the human sequence position, wherein said antibody or Fc fusion has specificity for a target antigen selected from the group consisting of CD20, CD22, CD33, CD52, Her2/neu, EGFR, EpCAM, MUC1, GD3, CEA, CA 125, HLA-DR, TNFalpha, and VEGF.

67. An antibody according to claims 61, 62, 63, 64, 65 or 66 wherein said Fc variant additionally comprises the substitutions 298A.

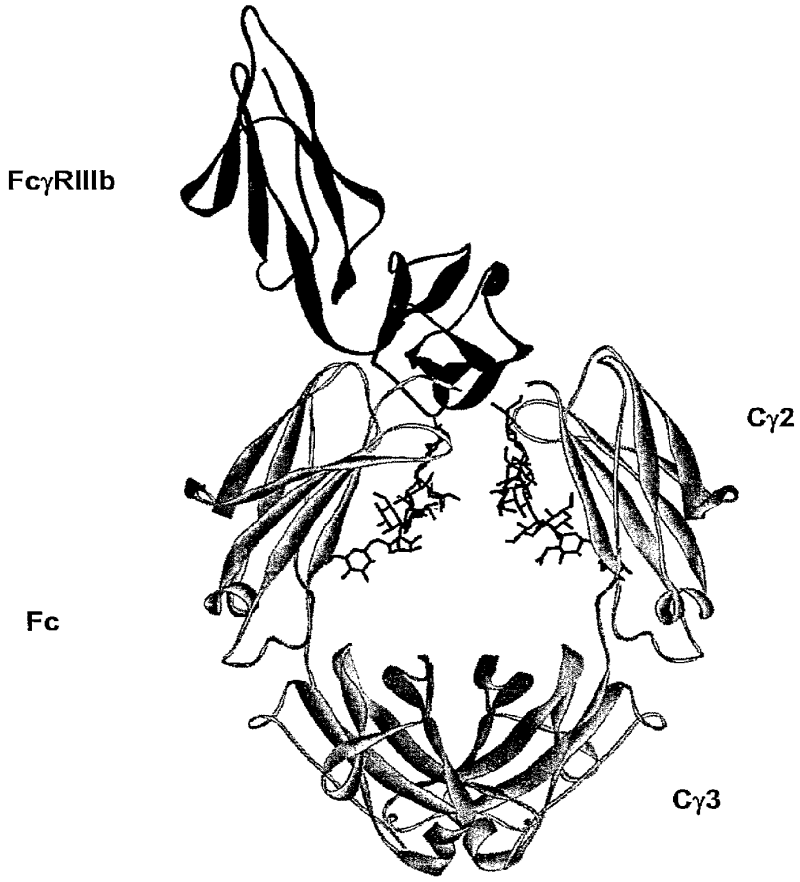
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Figure 1



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Figure 2



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Figure 3

```
VH1->
1        10         20         30         40         50         60
12345678901234567890123456789012345678901234567890123456789012345678901234567890
QVQLQESGPGLVRPSQTLSLTCTVSGFTFTDFYMNWVRQPPGRGLEWIGFIRDKAKGYTT

         70         80         90        100        110        120
12345678901234567890123456789012345678901234567890123456789012345678901234567890
EYNPSVKGRVTMLVDTSKNQFSLRLSSVTAADTAVYYCAREGHTAAPFDYWGQGSLVTVS

  Cγ1->
       130        140        150        160        170        180
12345678901234567890123456789012345678901234567890123456789012345678901234567890
SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS

                                                  HINGE->
       190        200        210        220        230        240
12345678901234567890123456789012345678901234567890123456789012345678901234567890
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG
                                                  1234567890123456
                                        KABAT  22        23

Cγ2->
       250        260        270        280        290        300
12345678901234567890123456789012345678901234567890123456789012345678901234567890
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
78901234567890123456789012345678901234567890123456789012345678901234567890123456
 240        250        260        270        280        290

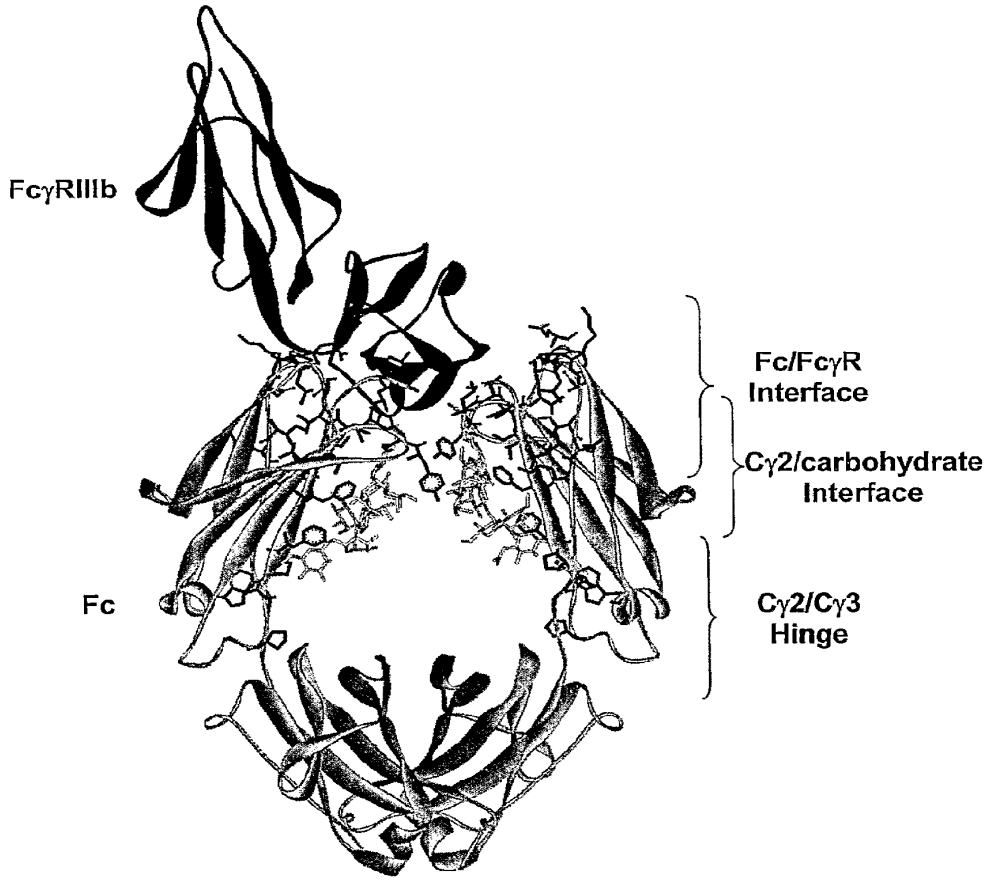
                                                          ?Cγ3?->
       310        320        330        340        350        360
12345678901234567890123456789012345678901234567890123456789012345678901234567890
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
78901234567890123456789012345678901234567890123456789012345678901234567890123456
 300        310        320        330        340        350

       370        380        390        400        410        420
12345678901234567890123456789012345678901234567890123456789012345678901234567890
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
78901234567890123456789012345678901234567890123456789012345678901234567890123456
 360        370        380        390        400        410

       430        440        450
123456789012345678901234567890123456789012345678901
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
78901234567890123456789012345678901234567890123456
 420        430        440
```

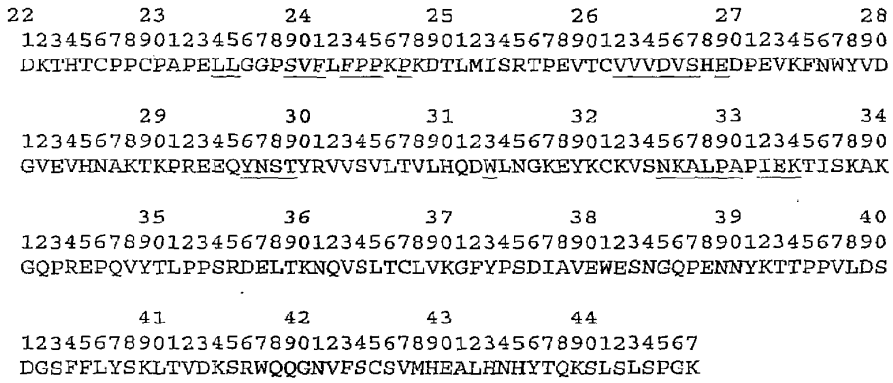

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Figure 4



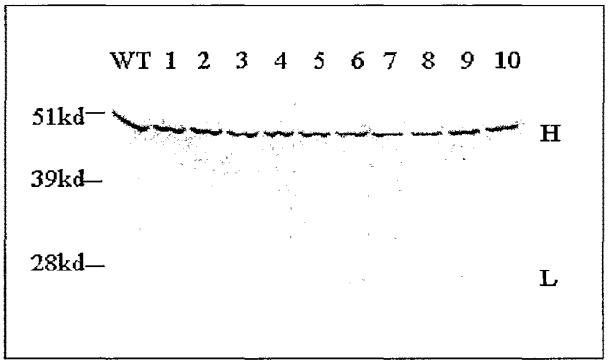
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Figure 5



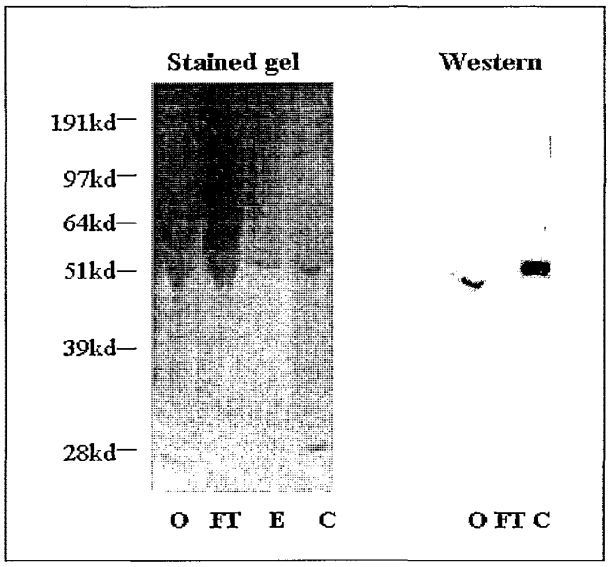
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Figure 6



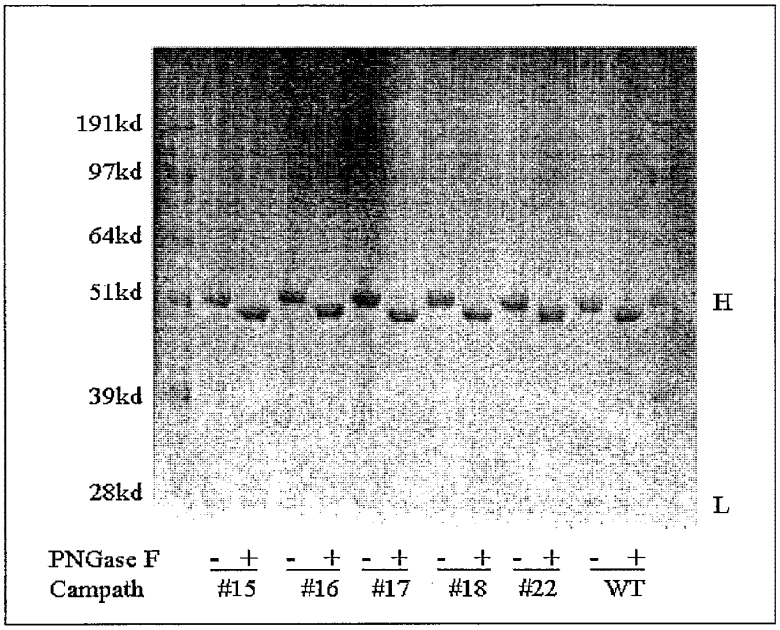
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Figure 7



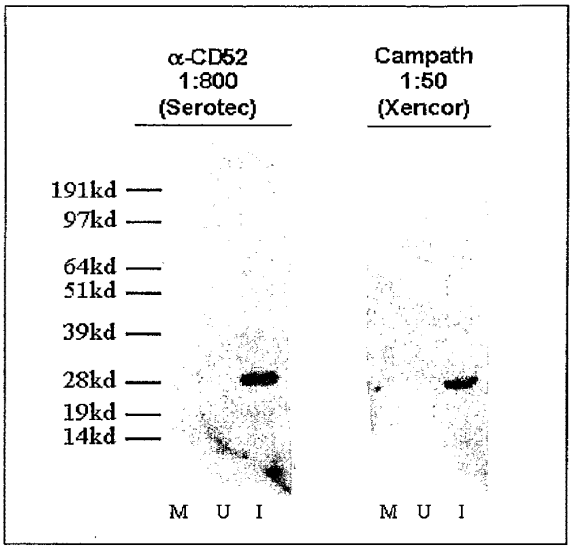
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Figure 8



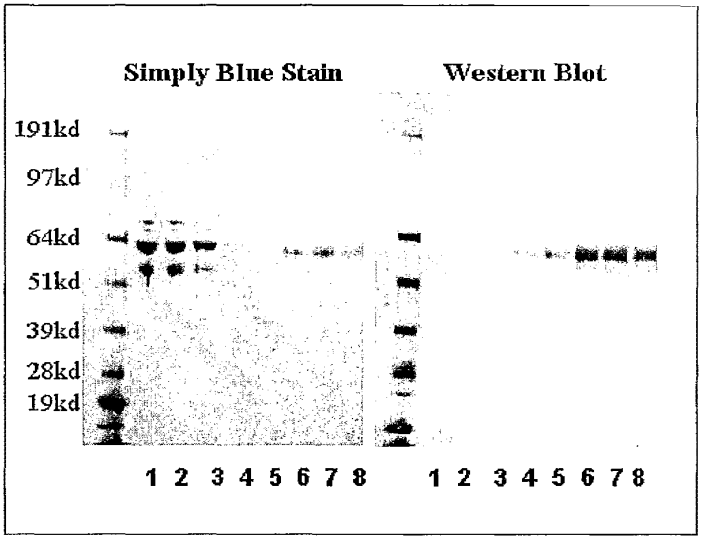
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Figure 9



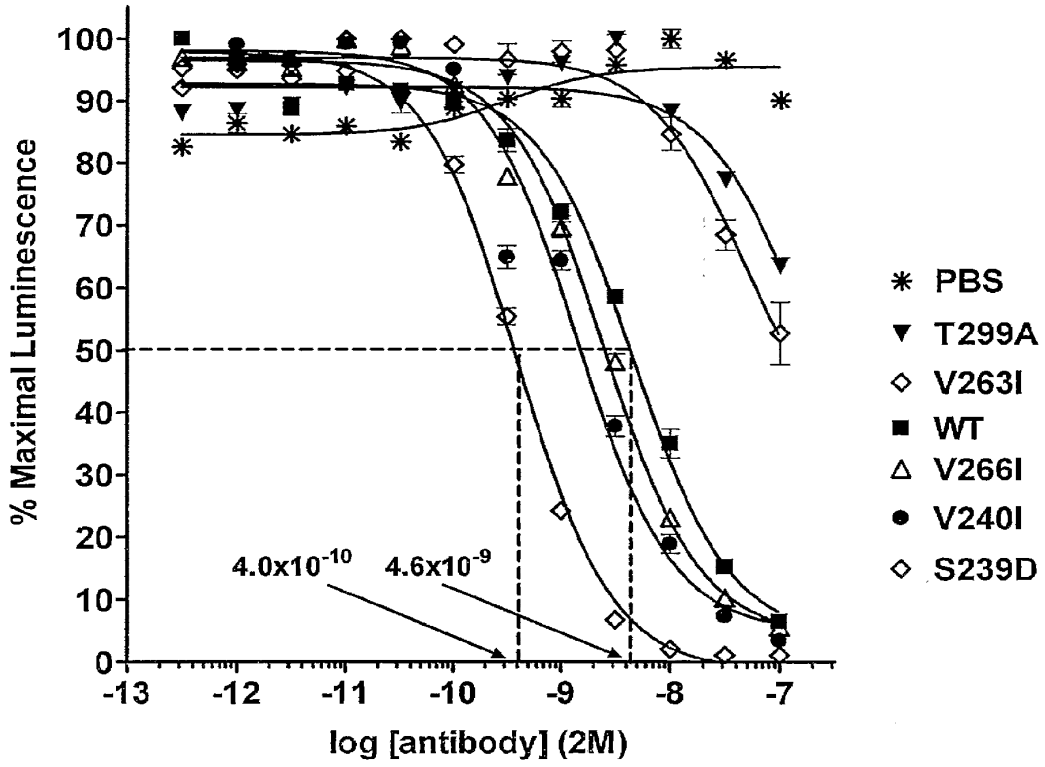
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Figure 10



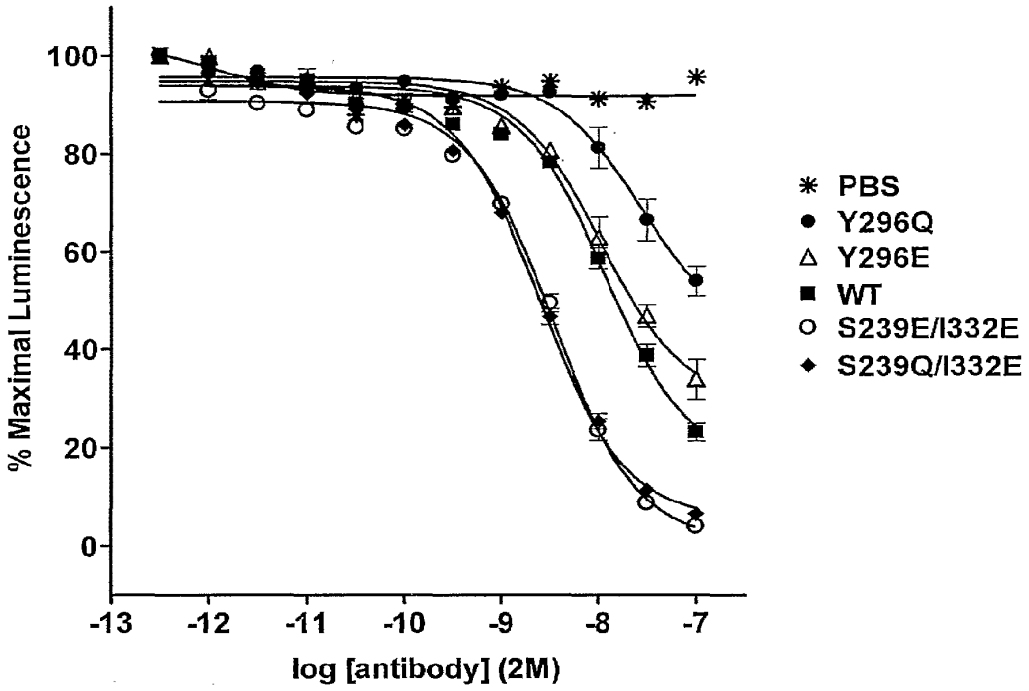
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Figure 11



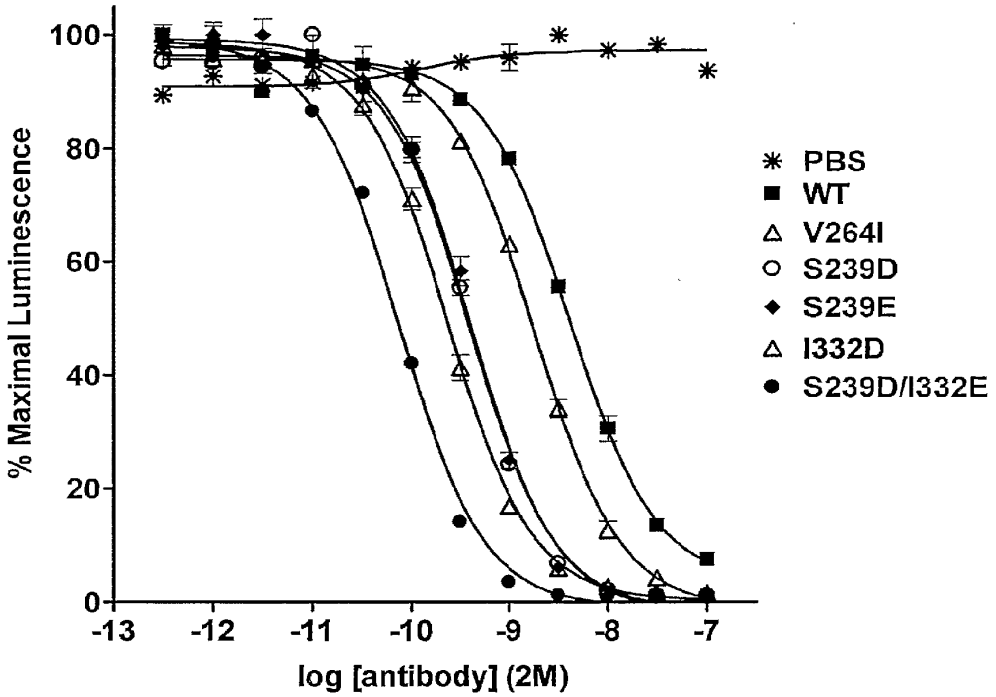
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Figure 12



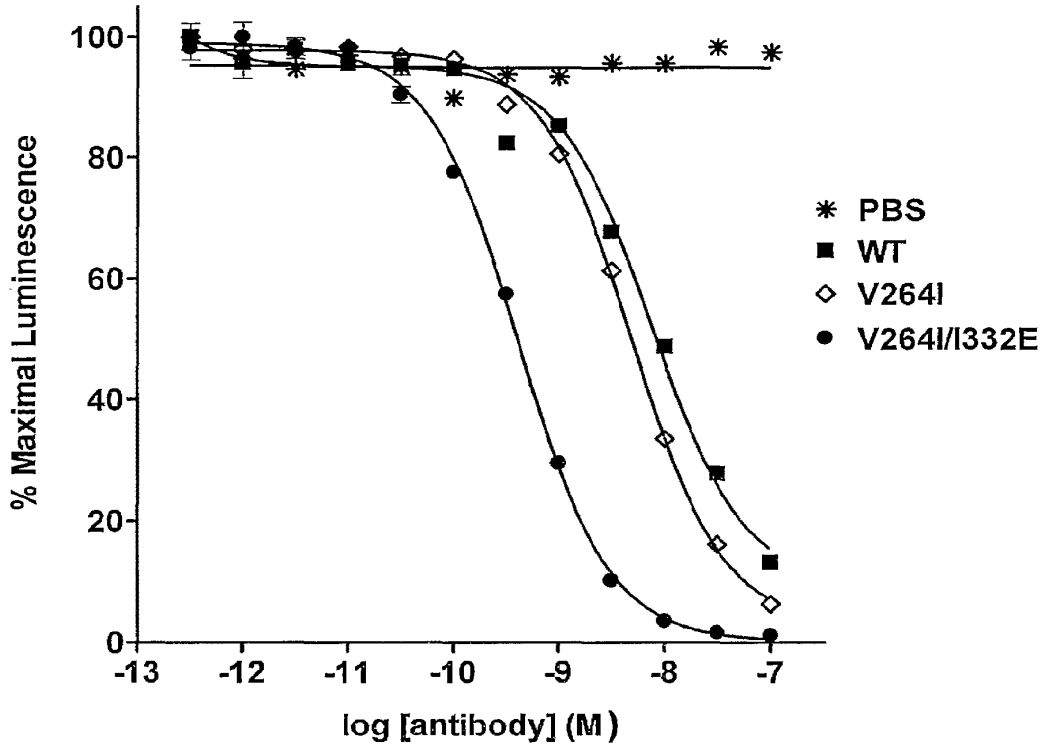
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Figure 13



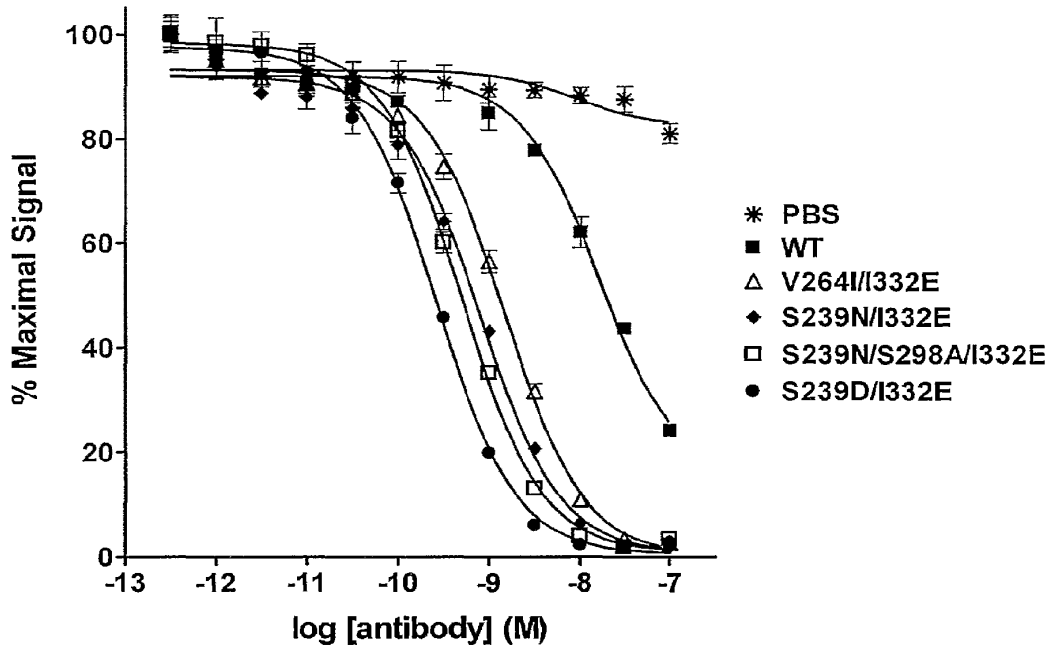
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Figure 14



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Figure 15



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Figure 16a

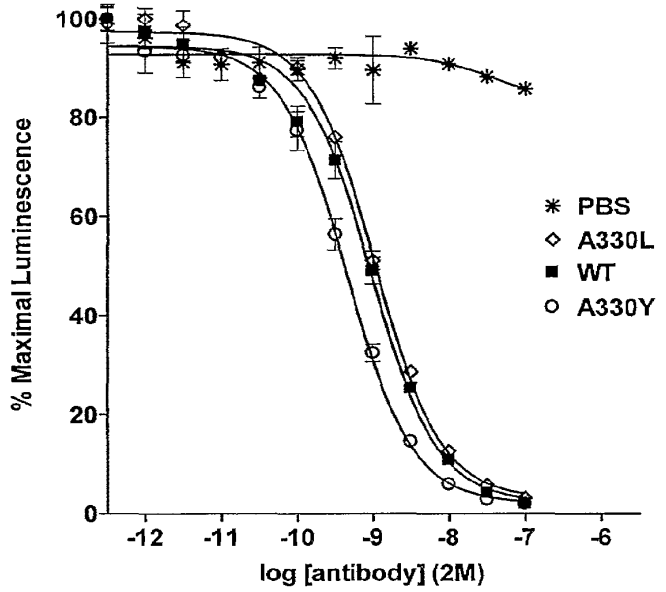
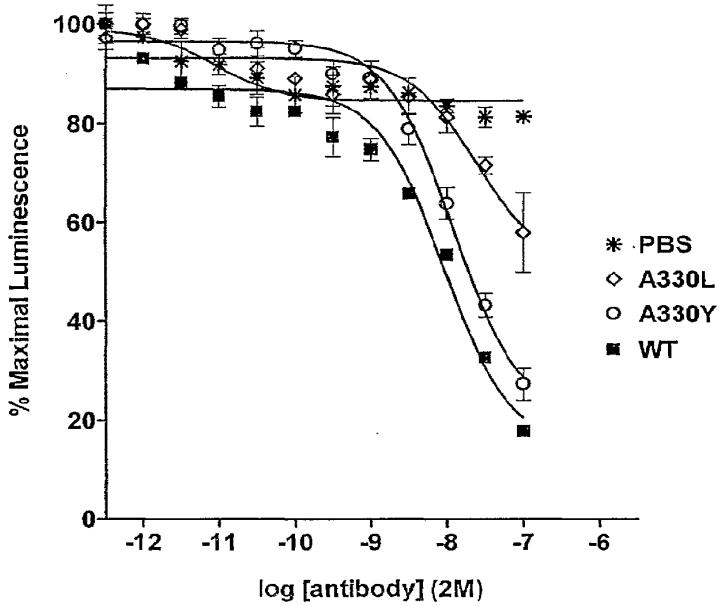
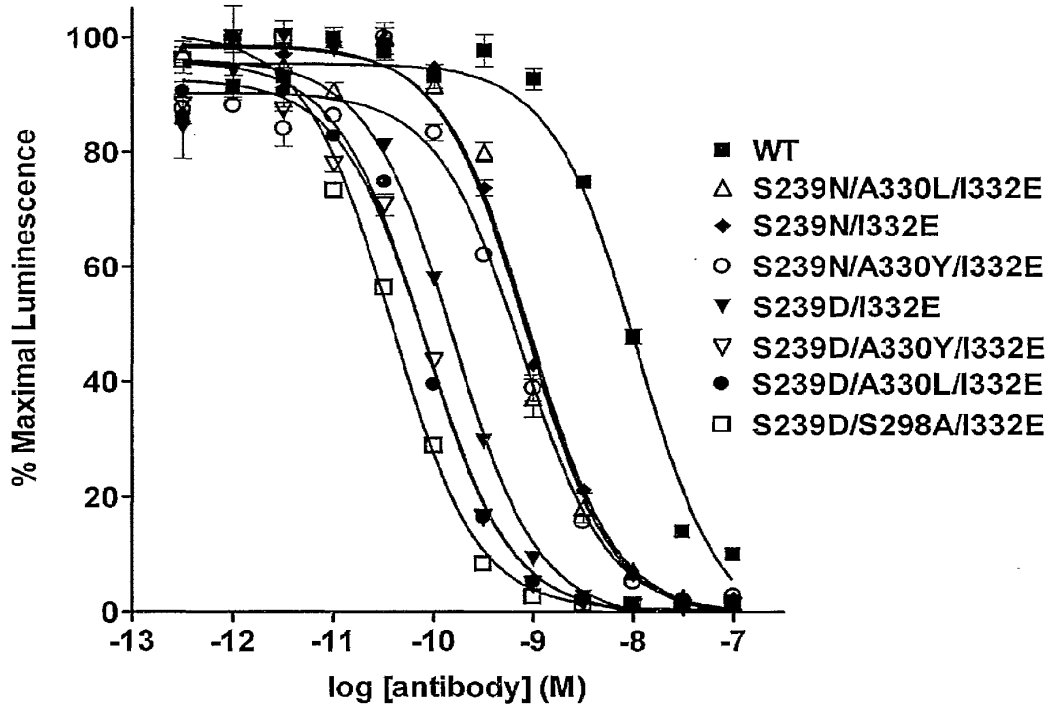


Figure 16b



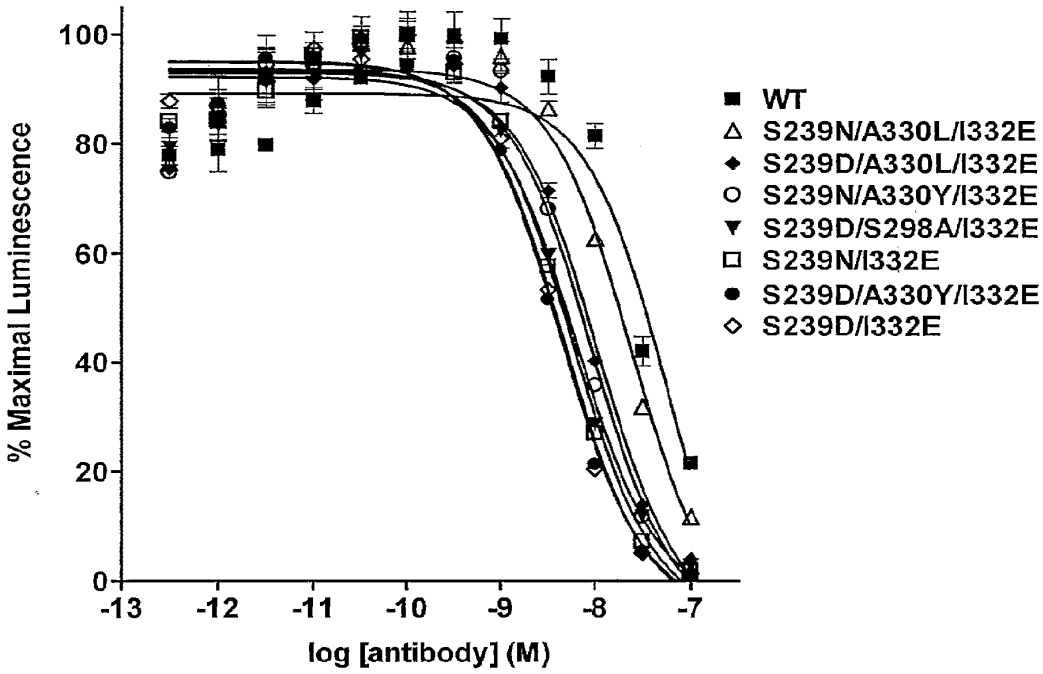
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Figure 17



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Figure 18



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Figure 19a

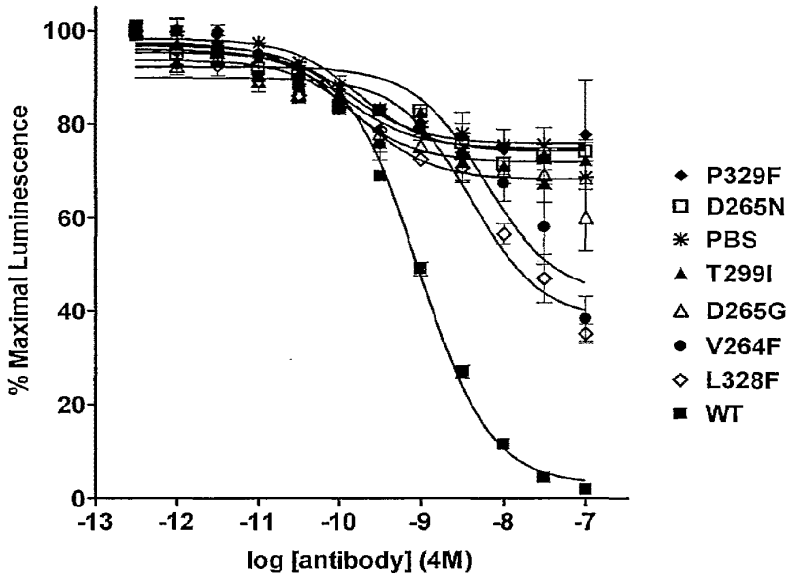
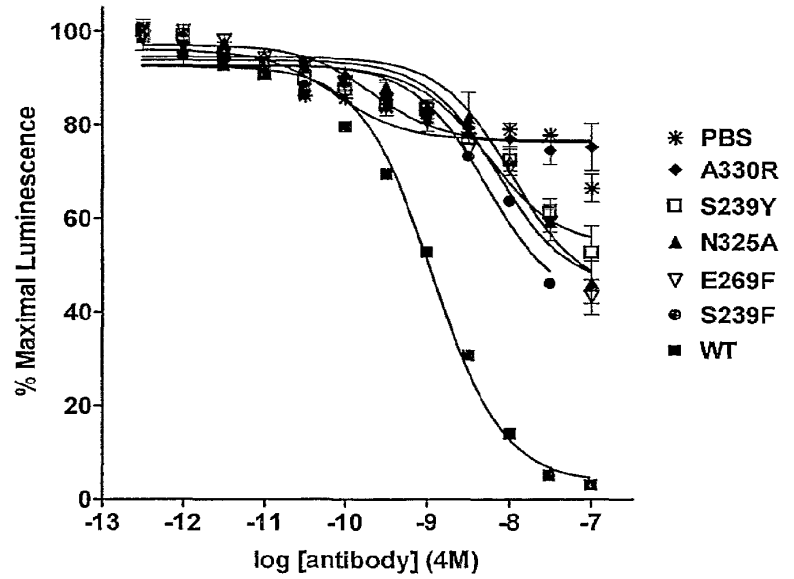
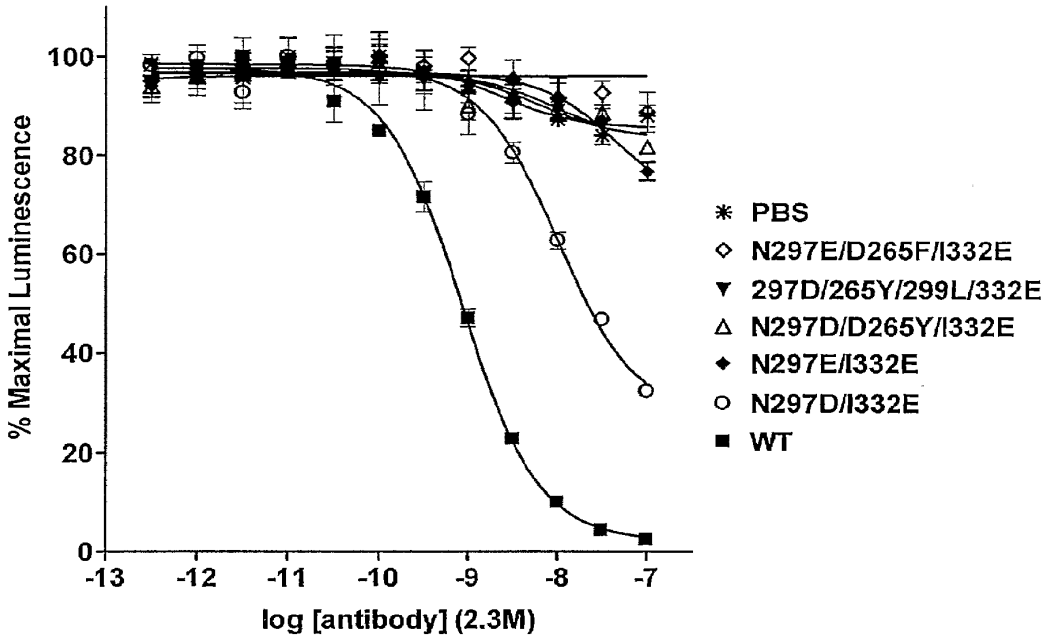


Figure 19b



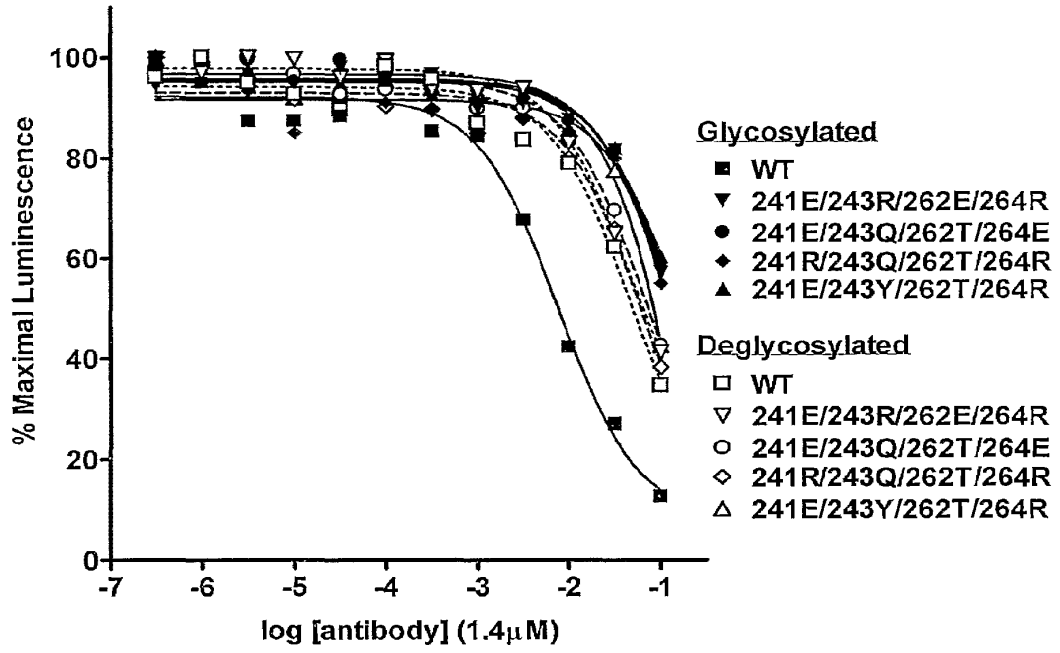
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Figure 20



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Figure 21



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Figure 22a

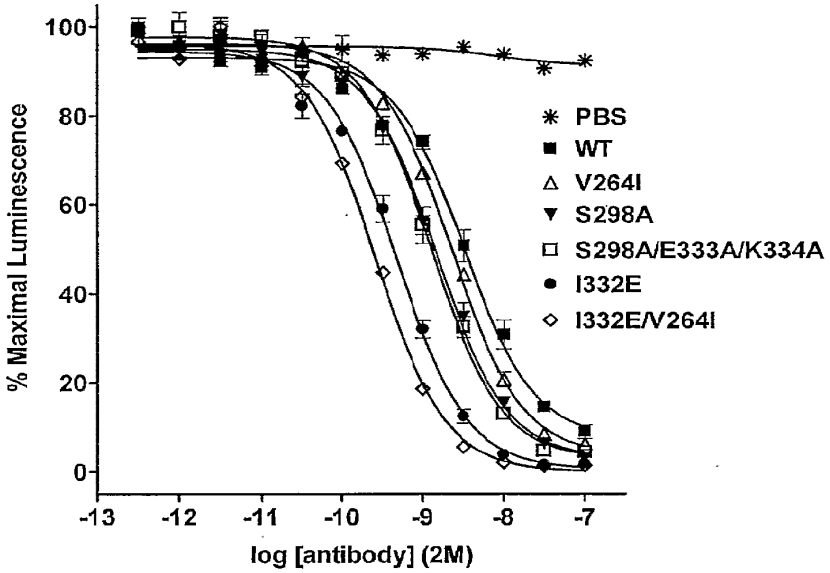
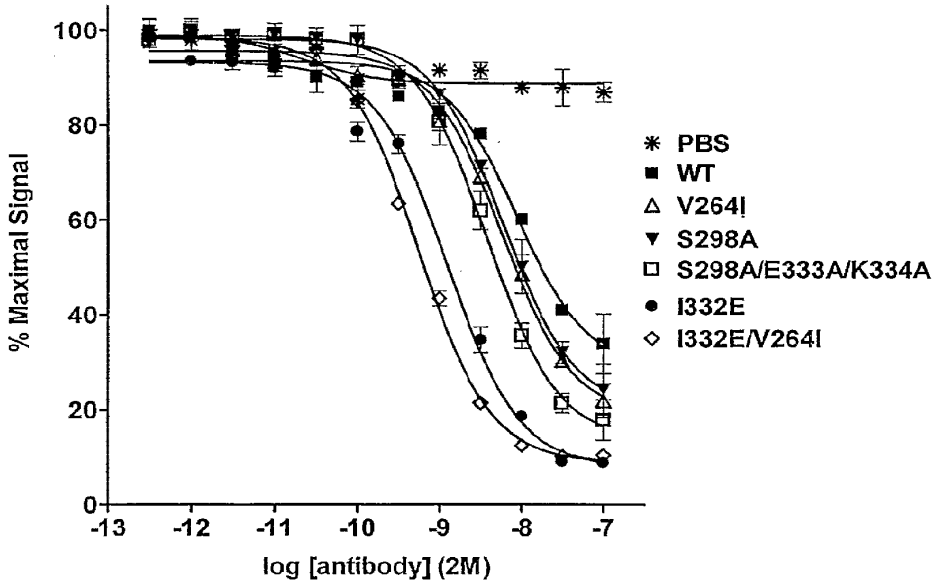


Figure 22b



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Figure 23a

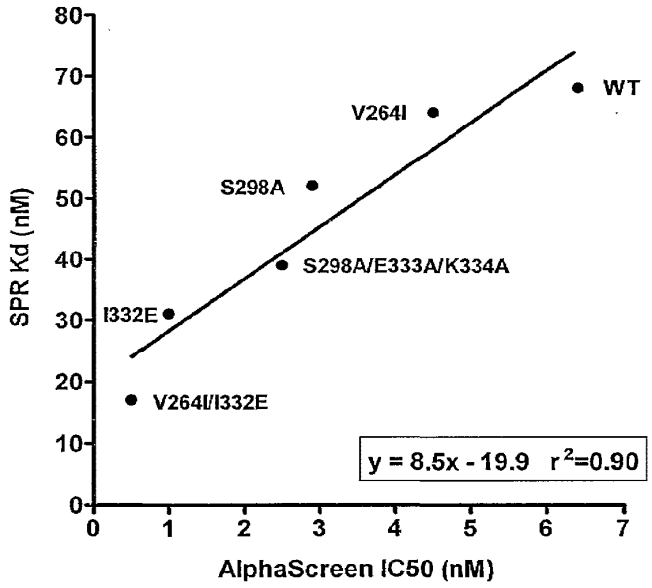
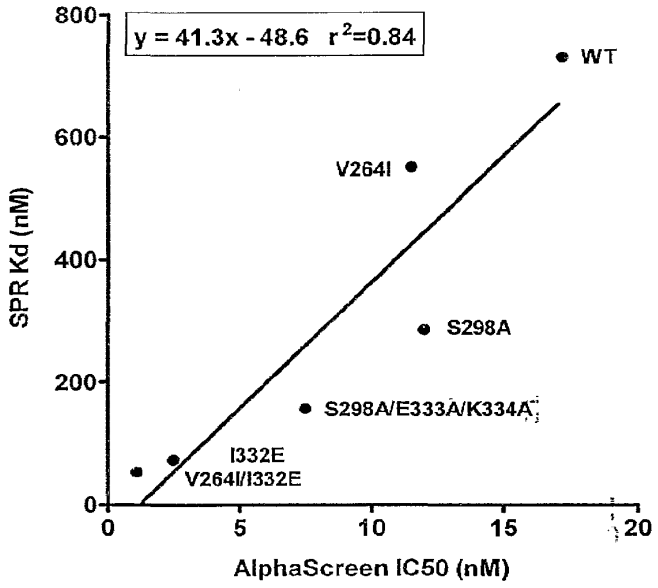


Figure 23b



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Figure 23c

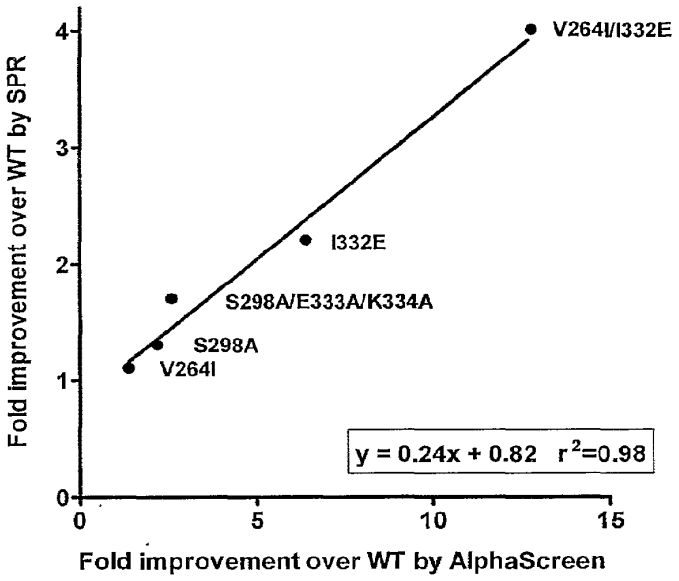
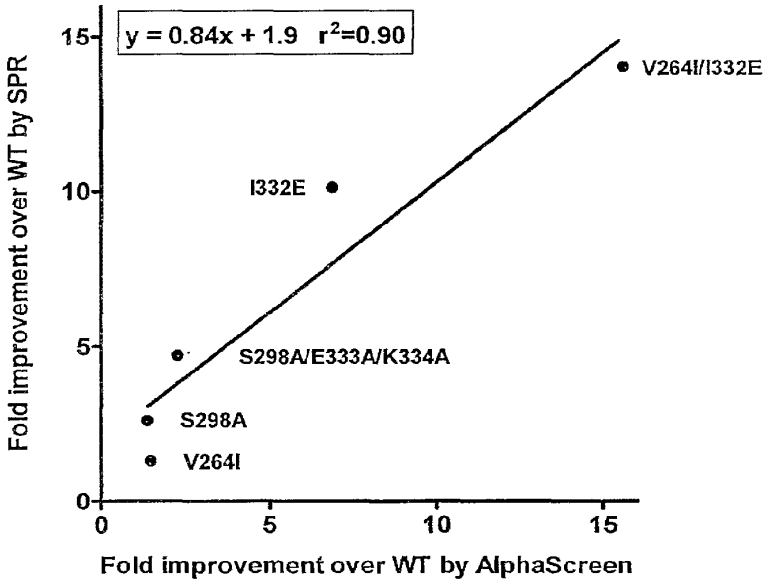
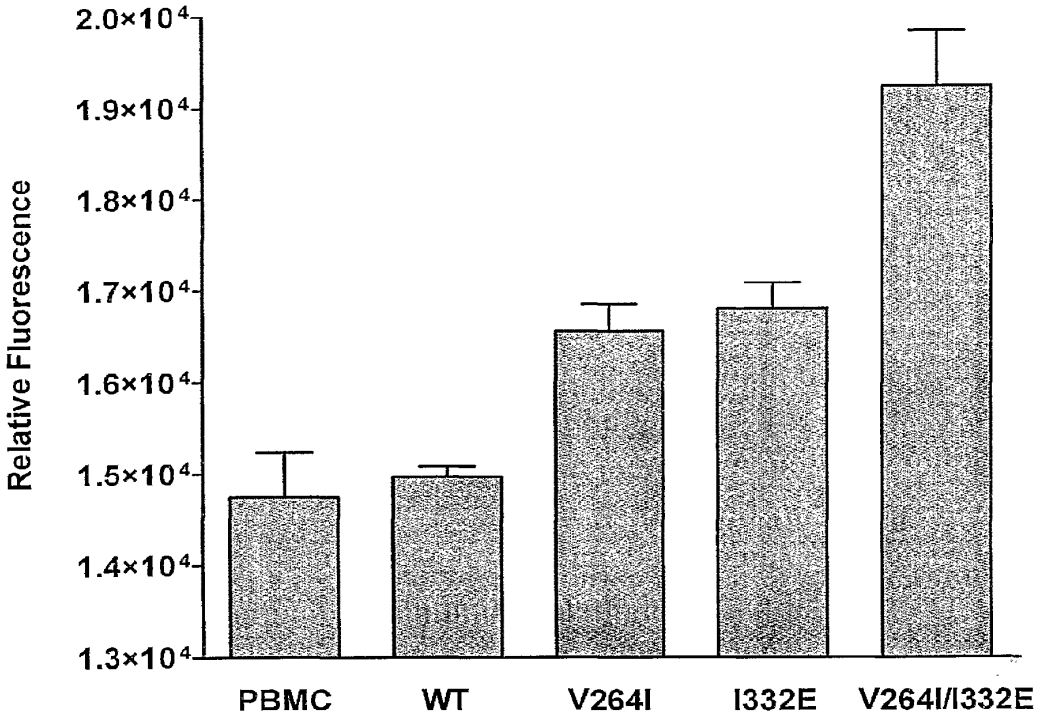


Figure 23d



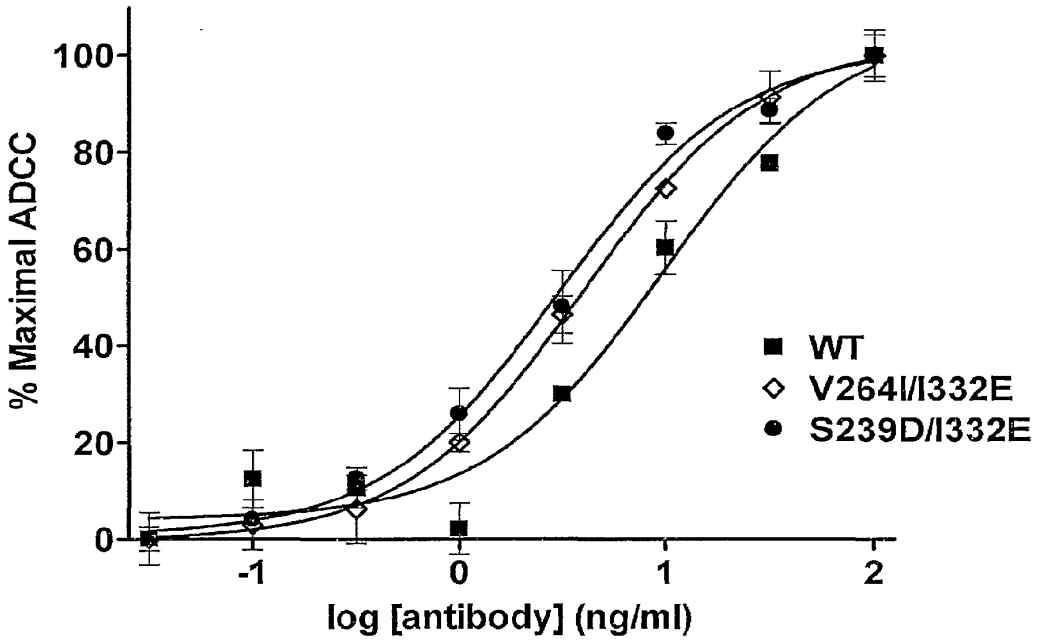
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Figure 24a



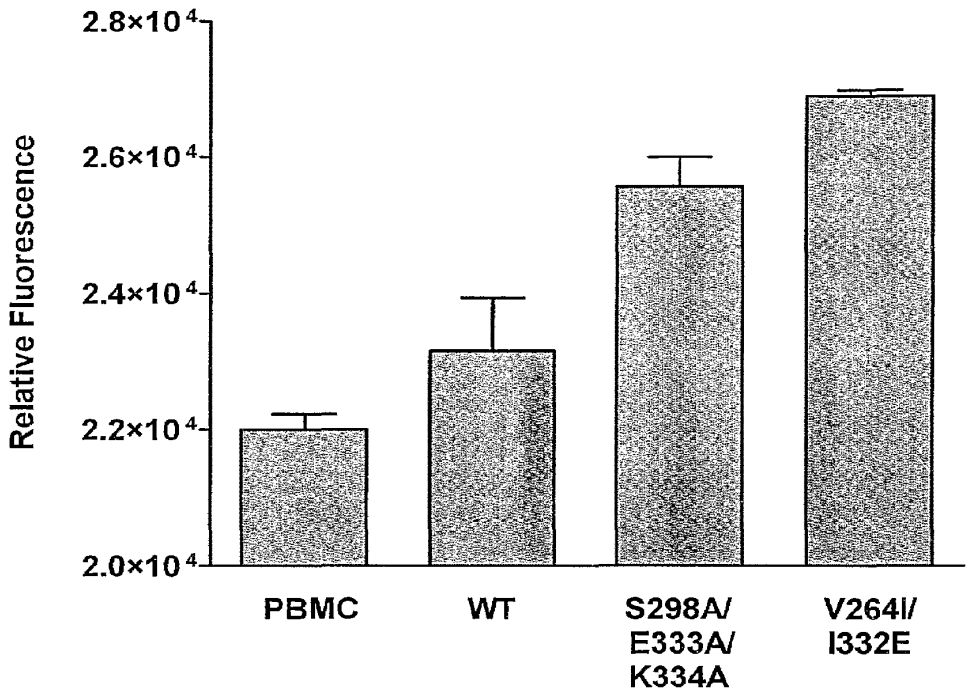
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Figure 24b



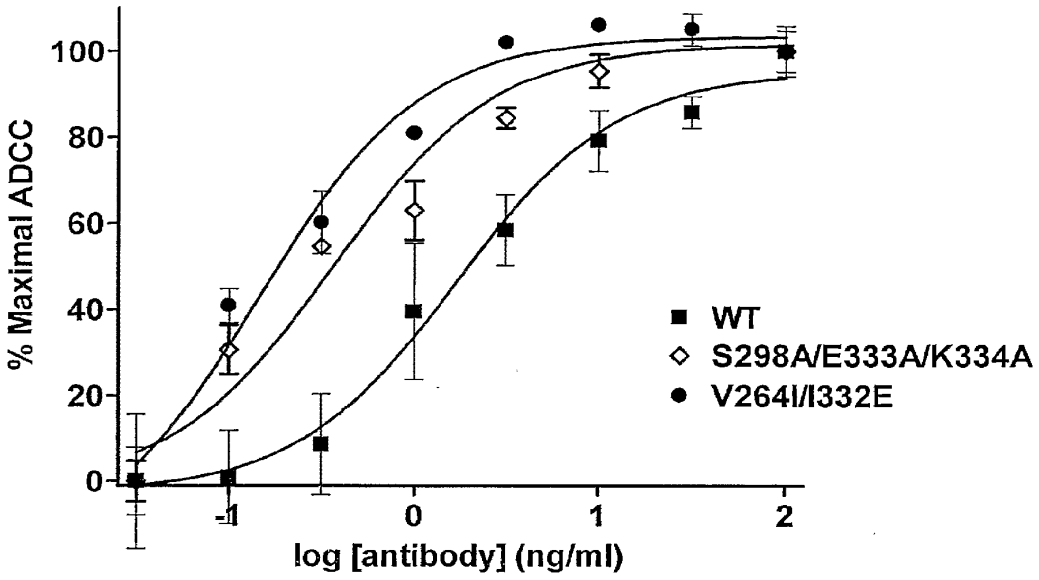
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Figure 25a



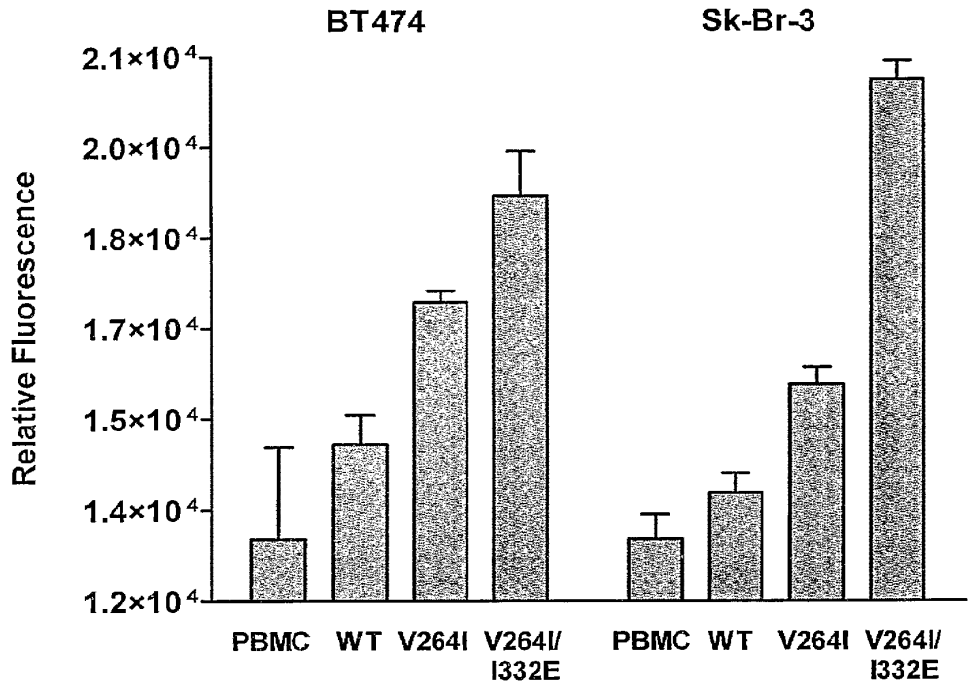
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Figure 25b



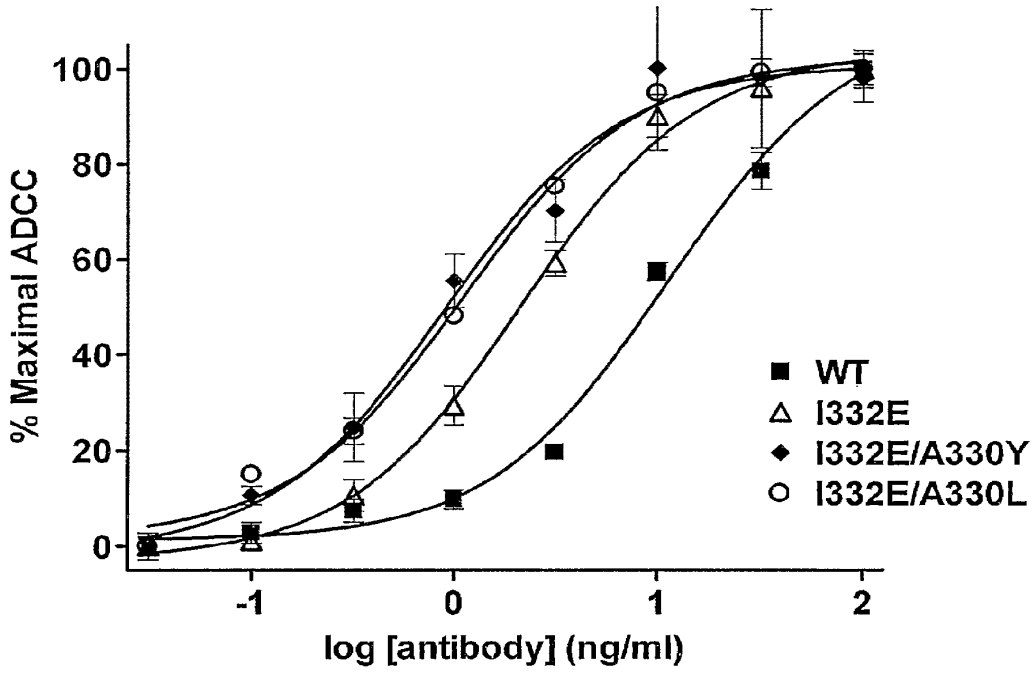
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Figure 26a



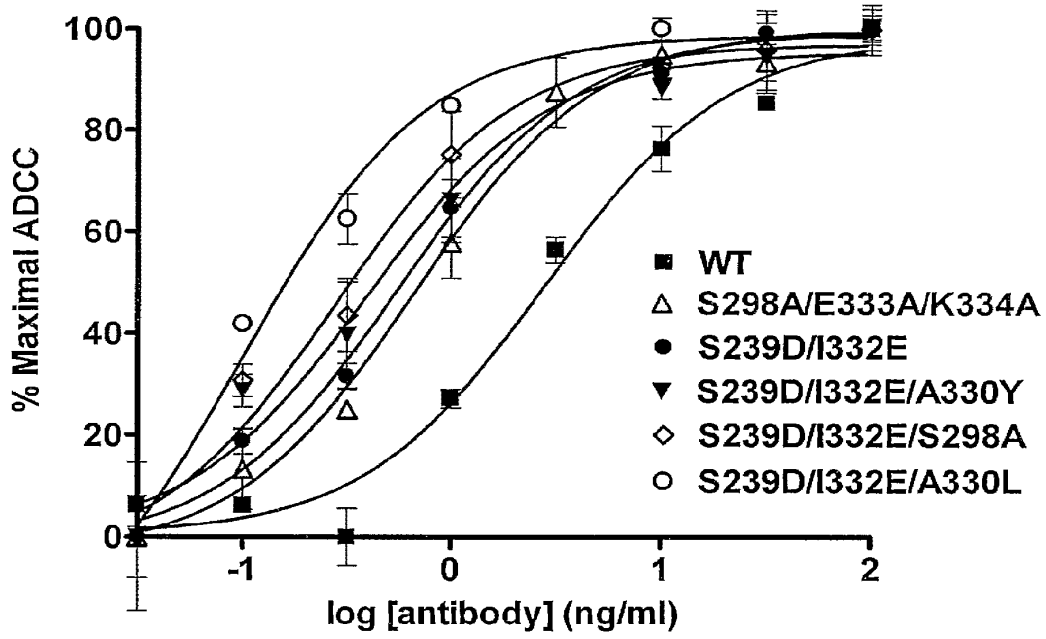
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Figure 26b



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Figure 26c



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Figure 27a

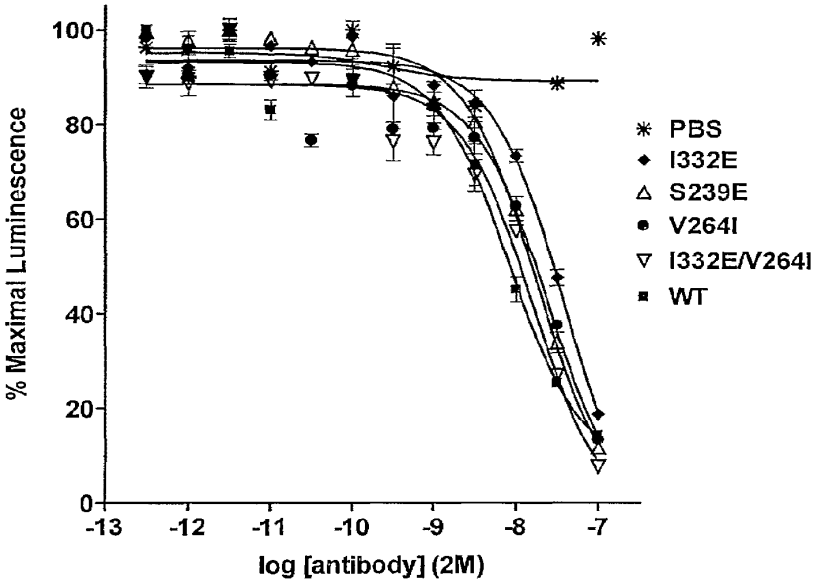
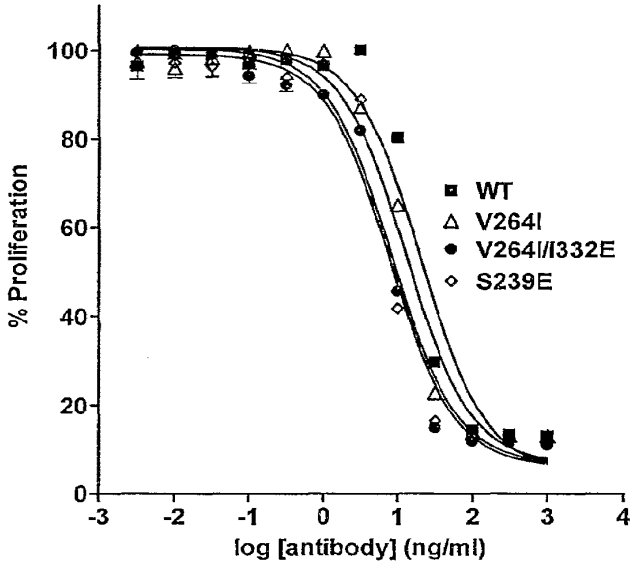
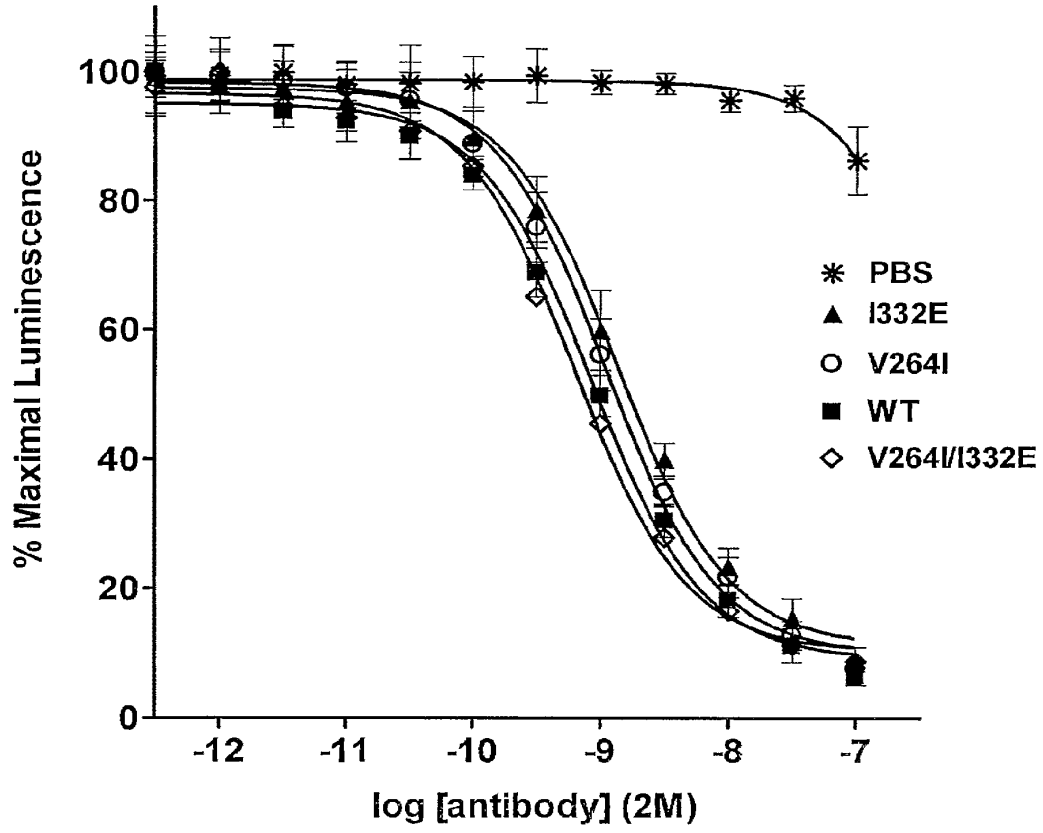


Figure 27b



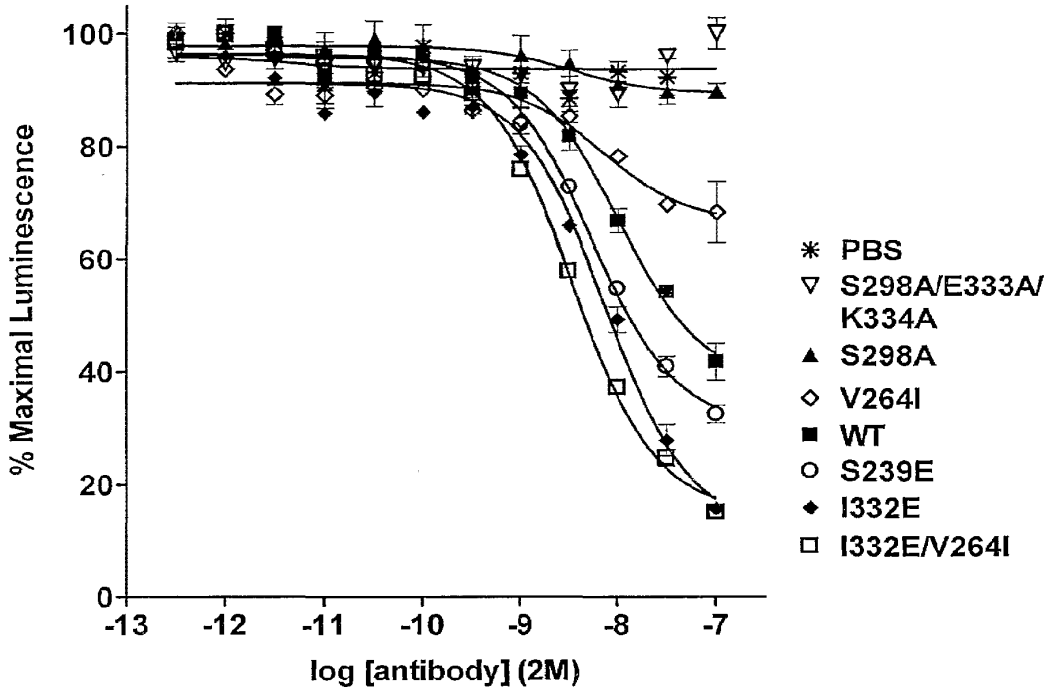
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Figure 28



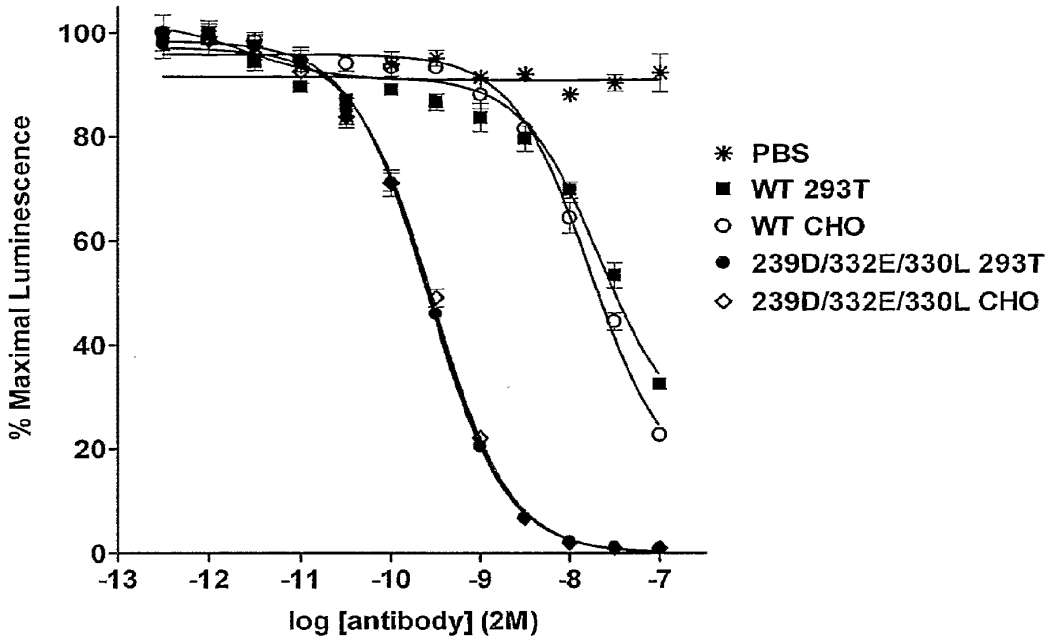
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Figure 29



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Figure 30



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Figure 31a

Anti-CD20 light chain
QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSG
TSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS
SPVTKSFNRGEC

Figure 31b

Anti-CD20 heavy chain

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFK
GKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSAASTKGPSVFP
LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
TQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGP**SV**FLFPPKPKDTLMISRTPEVTCV
VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**NS**TYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LP**A**PIEKTISKAKGQPREPQVYTLPP**S**RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Figure 31c

Anti-CD20 heavy chain comprising possible Fc variants
QVQLQQPGAELVKPGA**S**VKMSCKASGYTFTSY**N**MHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFK
GKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYF**N**VWGAGTTVTVSAASTKGPSVFP
LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
TQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGP**X_1X_2**FLFPPKPKDTLMISRTPEVTC
VV**X_3**DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**X_4Z_1**TYRVVSVLTVLHQDWLNGKEYKCKVSN
KALP**X_5PX_6**EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Position	WT	Possible Substitutions
X_1	S	D, E, N, Q, T
X_2	V	I, M
X_3	V	I, T, Y
X_4	N	D
X_5	A	Y, L, I
X_6	I	D, E, N, Q
Z_1	S	A

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BELL, Leonard** [US/US]; 59 Tumblebrook Road, Woodbridge, CT 06525 (US). **ROTHER, Russell, P.** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US).

(74) Agents: **DELUCA, Peter** et al.; Carter, DeLuca, Farrell & Schmidt, LLP, 445 Broadhollow Road, Suite 225, Melville, NY 11747-3615 (US).

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(54) Title: METHOD OF TREATING HEMOLYTIC DISEASE

Biochemical Parameters of Hemolysis During Eculizumab Treatment

Biochemical Marker	Normal range	Time of Analysis			p-value[a]
		Pre-study[b]	12 weeks	64 weeks	
LDH (IU/L)	150 - 480	3110.7 +/- 598.4	594.0 +/- 31.7	622.4 +/-41.1	0.002
AST (IU/L)	10 - 40	76.2 +/- 16.0	26.2 +/- 2.3	30.1 +/- 3.2	0.02
Haptoglobin (g/L)	0.5 - 2	<0.06	<0.06	0.14 +/- 0.07[c]	N.S.[d]
Hemoglobin (g/dL)	11.5 - 18	10.0 +/- 0.4	10.3 +/- 0.4	10.4 +/- 0.4	N.S.
Bilirubin	3 - 15	25.9 +/- 4.3	28.2 +/- 4.4	28.7 +/- 4.0	N.S.
Reticulocytes (x10^{-3}/mm^3)	20 - 80	161.4 +/- 25.9	191.2 +/- 23.6	189.6 +/- 21.8	N.S.

[a]from comparisons of mean change from pre-study to 64 weeks
[b]values represent means during 52 week period prior to treatment except for AST which represents the baseline mean
[c]10 of 11 patients were below the detectable limit of haptoglobin (<0.06 g/L); 1 patient had a value of 0.69 g/L.
[d]not significant

(57) Abstract: Paroxysmal nocturnal hemoglobinuria or other hemolytic diseases are treating using a compound which binds to or otherwise blocks the generation and/or the activity of one or more complement components, such as, for example, a complement-inhibiting antibody.

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METHOD OF TREATING HEMOLYTIC DISEASE

RELATED APPLICATIONS

This application claims priority to, and is a continuation-in-part of, U.S. Patent Application Serial No. 10/771,552, filed February 3, 2004, the entire disclosure of which is incorporated herein by this reference.

BACKGROUND

Technical Field

This disclosure relates to a method of treating a hemolytic disease such as, for example, paroxysmal nocturnal hemoglobinuria ("PNH"), by administering a compound which binds to, or otherwise blocks, the generation and/or activity of one or more complement components.

Background of Related Art

Paroxysmal nocturnal hemoglobinuria ("PNH") is an uncommon blood disorder wherein red blood cells are compromised and are thus destroyed more rapidly than normal red blood cells. PNH results from a mutation of bone marrow cells resulting in the generation of abnormal blood cells. More specifically, PNH is believed to be a disorder of hematopoietic stem cells, which give rise to distinct populations of mature blood cells. The basis of the disease appears to be somatic mutations leading to the inability to synthesize the glycosyl-phosphatidylinositol ("GPI") anchor that is responsible for binding

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proteins to cell membranes. The mutated gene, PIG-A (phosphatidylinositol glycan class A) resides in the X chromosome and can have several different mutations, varying from deletions to point mutations.

PNH causes a sensitivity to complement proteins and this sensitivity occurs in the cell membrane. PNH cells are deficient in a number of proteins, particularly essential complement-regulating surface proteins. These complement-regulating surface proteins include the decay-accelerating factor ("DAF") or CD55 and membrane inhibitor of reactive lysis ("MIRL") or CD59.

PNH is characterized by hemolytic anemia (a decreased number of red blood cells), hemoglobinuria (the presence of hemoglobin in the urine particularly evident after sleeping), and hemoglobinemia (the presence of hemoglobin in the bloodstream). PNH-afflicted individuals are known to have paroxysms, which are defined here as incidences of dark-colored urine. Hemolytic anemia is due to intravascular destruction of red blood cells by complement components. Other known symptoms include dysphagia, fatigue, erectile dysfunction, thrombosis and recurrent abdominal pain.

Hemolysis resulting from hemolytic diseases causes local and systemic nitric oxide (NO) deficiency through the release of free hemoglobin. Free hemoglobin is a very efficient scavenger of NO, due in part to the accessibility of NO in the non-erythrocyte compartment and a 10^6 times greater affinity of the heme moiety for NO than that for oxygen. The occurrence of intravascular hemolysis often generates sufficient free hemoglobin to completely deplete haptoglobin. Once the capacity of this hemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues. For example, in a setting of intravascular hemolysis such as PNH, where LDH levels can easily exceed

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2 - 3 times their normal levels, free hemoglobin would likely obtain concentrations of 0.8 – 1.6 g/l. Since haptoglobin can only bind somewhere between 0.7 to 1.5 g/l of hemoglobin depending on the haptoglobin allotype, a large excess of free hemoglobin would be generated. Once the capacity of hemoglobin reabsorption by the kidney proximal tubules is exceeded, hemoglobinuria ensues. The release of free hemoglobin during intravascular hemolysis results in excessive consumption of NO with subsequent enhanced smooth muscle contraction, vasoconstriction and platelet activation and aggregation. PNH-related morbidities associated with NO scavenging by hemoglobin include abdominal pain, erectile dysfunction, esophageal spasm, and thrombosis.

The laboratory evaluation of hemolysis normally includes hematologic, serologic, and urine tests. Hematologic tests include an examination of the blood smear for morphologic abnormalities of RBCs (to determine causation), and the measurement of the reticulocyte count in whole blood (to determine bone marrow compensation for RBC loss). Serologic tests include lactate dehydrogenase (LDH; widely performed), and free hemoglobin (not widely performed) as a direct measure of hemolysis. LDH levels, in the absence of tissue damage in other organs, can be useful in the diagnosis and monitoring of patients with hemolysis. Other serologic tests include bilirubin or haptoglobin, as measures of breakdown products or scavenging reserve, respectively. Urine tests include bilirubin, hemosiderin, and free hemoglobin, and are generally used to measure gross severity of hemolysis and for differentiation of intravascular vs. extravascular etiologies of hemolysis rather than routine monitoring of hemolysis. Further, RBC numbers, RBC (i.e. cell-

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bound) hemoglobin, and hematocrit are generally performed to determine the extent of any accompanying anemia rather than as a measure of hemolytic activity per se.

Steroids have been employed as a therapy for hemolytic diseases and may be effective in suppressing hemolysis in some patients, although long term use of steroid therapy carries many negative side effects. Afflicted patients may require blood transfusions, which carry risks of infection. Anti-coagulation therapy may also be required to prevent blood clot formation. Bone marrow transplantation has been known to cure PNH, however, bone marrow matches are often very difficult to find and mortality rates are high with such procedure.

It would be advantageous to provide a treatment which safely and reliably eliminates and/or limits hemolytic diseases, such as PNH, and their effects.

Summary

Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Suitable compounds include, for example, antibodies which bind to or otherwise block the generation and/or activity of one or more complement components, such as, for example, an antibody specific to complement component C5. In particularly useful embodiments, the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb (eculizumab), h5G1.1-scFv (pexelizumab) and other functional

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fragments of h5G1.1. It has surprisingly been found that the present methods provide improvements in the PNH subject within 24 hours of administration of the compound. For example, hemolysis is significantly reduced within 24 hours of administration of the compound as indicated by resolution of hemoglobinuria.

The complement-inhibiting compound can be administered prophylactically in individuals known to have a hemolytic disease to prevent, or help prevent the onset of symptoms. Alternatively, the complement-inhibiting compound can be administered as a therapeutic regimen to an individual experiencing symptoms of a hemolytic disease.

In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. The method comprises administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. By increasing type III red blood cell count, symptoms such as fatigue and anemia also can be alleviated in a patient afflicted with a hemolytic disease.

In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

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It has surprisingly been found that patients can be rendered transfusion-independent in accordance with the present methods. Unexpectedly, transfusion-independence can be maintained in some embodiments for twelve months or more, long beyond the 120 day life cycle of red blood cells. In other embodiments, transfusion-independence can be maintained for two years or more. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells.

In another aspect, the present disclosure contemplates a method of treating a nitric oxide (NO) imbalance in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of nitric oxide (NO). In particularly useful embodiments, NO homeostasis is restored wherein there is a resolution of symptoms attributable to NO deficiency.

In another aspect, the present disclosure contemplates a method of treating thrombosis in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

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In another aspect, the present disclosure contemplates a method of treating fatigue in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

In another aspect, the present disclosure contemplates a method of treating erectile dysfunction in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

In another aspect, the present disclosure contemplates a method of treating abdominal pain in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

In yet another aspect, the present disclosure contemplates a method of treating a subject afflicted with a hemolytic disease by administering: 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell

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inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject where the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10% before or during treatment, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered

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alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject having a platelet count above 40,000 per microliter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of a hemolytic diseases in a subject having a reticulocyte count above 80 x 10^9 per liter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

Brief Description of the Drawings

Figure 1A reports biochemical parameters of hemolysis measured during treatment of PNH patients with an anti-C5 antibody.

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Figure 1B graphically depicts the effect of treatment with an anti-C5 antibody on lactate dehydrogenase (LDH) levels.

Figure 2 shows a urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in PNH patients.

Figure 3 is a graph of the effects of eculizumab treatments on patient paroxysm rates, as compared to pre-treatment rates.

Figure 4 shows urine samples of PNH patients and measurements of hemoglobinuria, dysphagia, LDH, AST, pharmacokinetics (PK) and pharmacodynamics (PD) reflecting the immediate and positive effects of the present methods on hemolysis, symptoms and pharmacodynamics suitable to completely block complement.

Figure 5 graphically depicts the effect of anti-C5 antibody dosing schedule on hemoglobinuria over time.

Figures 6a and 6b are graphs comparing the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody: Figure 6a depicts cytopenic patients; and Figure 6b depicts non-cytopenic patients.

Figure 7 shows the management of a thrombocytopenic patient by administering an anti-C5 antibody and erythropoietin (EPO).

Figure 8 graphically depicts the pharmacodynamics of an anti-C5 antibody.

Figure 9 is a chart of the results of European Organization for Research and Treatment of Cancer questionnaires ("EORTC QLC-C30") completed during the anti-C5 therapy regimen addressing quality of life issues.

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Figure 10 is a chart depicting the effects of anti-C5 antibody treatments on adverse symptoms associated with PNH.

Detailed Description

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the

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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.

In another embodiment, soluble forms of the proteins CD55 and CD59, singularly or in combination with each other, can be administered to a subject to inhibit the complement cascade in its alternative pathway. CD55 inhibits at the level of C3, thereby preventing the further progression of the cascade. CD59 inhibits the C5b-8 complex from combining with C9 to form the membrane attack complex (see discussion below).

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of bacterial and viral pathogens. There are at least 25 proteins involved in the complement cascade, which are found as a complex collection of plasma proteins and membrane cofactors. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with

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complement activation is provided, for example, in The Merck Manual, 16th Edition.

The complement cascade progresses via the classical pathway, the alternative pathway or the lectin pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells. The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin ("MBL") to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease to yield C3a and C3b.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacteria and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

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C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to all three activation pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b (inactive C3b). While still functional as an opsonin, iC3b can not form an active C5 convertase.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the sequence), with four amino acids (amino acid residues 656-659 of the sequence) deleted between the two. C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 655 amino acid 75 kDa beta chain. C5 is found in normal serum at approximately 75 µg/ml (0.4 µM). C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1658 amino acids along with an 18 amino acid leader sequence (see, U.S. patent 6,355,245).

Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74

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amino acids of the alpha chain (i.e., amino acid residues 660-733 of the sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the sequence. A compound that binds at, or adjacent, to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

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

C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and various cytokines. C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau

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et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

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

Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components may be utilized in accordance with the present disclosure. In some embodiments, antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2, thus preventing the generation of the anaphylatoxic activity associated with C5a and/or preventing the assembly of the membrane attack complex associated with C5b.

Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available

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from Archemix Corp., Cambridge, MA), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

Functionally, one suitable class of compounds inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the compound does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

While preventing the generation of these membrane attack complex molecules, inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for hemolytic diseases, where increased susceptibility to thrombosis, infection, fatigue, lethargy and impaired clearance of immune complexes are pre-existing clinical features of the disease process.

Particularly useful compounds for use herein are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antigen binding site and exhibiting specific binding to human complement component C5. Particularly useful complement inhibitors are compounds which reduce the generation of C5a

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and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. 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.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement C5 component as immunogens according to known methods. In accordance with this disclosure, complement component

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C5, C5a or C5b is preferably used as the immunogen. In accordance with particularly preferred useful embodiments, the immunogen is the alpha chain of C5.

Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete for binding to portions of C5 that are specifically immunoreactive with 5G1.1;

(2) they specifically bind to the C5 alpha chain -- such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198); and

(3) they block the binding of C5 to either C3 or C4 (which are components of the C5 convertases).

The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as fragments (e.g., Fab, $F(ab')_2$, scFv) will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

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

Administration of the compound that inhibits the production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier, via intravenous infusion by injection, subcutaneous injection, orally, or sublingually. Other routes of administration may be used if desired.

It is further contemplated that a combination therapy can be used wherein a complement-inhibiting compound is administered in combination with a regimen of known therapy for hemolytic disease. Such regimens include administration of 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In

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particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The present disclosure contemplates methods of reducing hemolysis in a patient afflicted with a hemolytic disease by administering one or more compounds which bind to or otherwise block the generation and/or activity of one or more complement components. Reducing hemolysis means that the duration of time a person suffers from hemolysis is reduced by about 25% or more. The effectiveness of the treatment can be evaluated in any of the various manners known to those skilled in the art for determining the level of hemolysis in a patient. One qualitative method for detecting hemolysis is to observe the occurrences of hemoglobinuria. Quite surprisingly, treatment in accordance with the present methods reduces hemolysis as determined by a rapid reduction in hemoglobinuria.

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A more qualitative manner of measuring hemolysis is to measure lactate dehydrogenase (LDH) levels in the patient's bloodstream. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. As those skilled in the art will appreciate, measurements of "upper limit of normal" levels of LDH will vary from lab to lab depending on a number of factors including the particular assay employed and the precise manner in which the assay is conducted. Generally speaking, however, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients to within 20% of the upper limit of normal LDH levels. Alternatively, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients of greater than 50% of the patient's pre-treatment LDH level, preferably greater than 65% of the patient's pre-treatment LDH level, most preferably greater than 80% of the patient's pre-treatment LDH level.

Another quantitative measurement of a reduction in hemolysis is the presence of GPI-deficient red blood cells (Type III red blood cells). As those skilled in the art will appreciate, Type III red blood cells have no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells can be determined by flow cytometry using, for example, the technique described in Richards, et al., *Clin. Appl. Immunol. Rev.*, vol. 1, pages 315-330, 2001. The present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by an increase in Type III red blood

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cells. Preferably an increase in Type III red blood cell levels in the patient of greater than 25% of the total red blood cell count is achieved, more preferably an increase in Type III red blood cell levels in the patients greater than 50% of the total red blood cell count is achieved, most preferably an increase in Type III red blood cell levels in the patients greater than 75% of the total red blood cell count is achieved.

Methods of reducing one or more symptoms associated with PNH or other hemolytic diseases are also within the scope of the present disclosure. Such symptoms include, for example, abdominal pain, fatigue, dyspnea and insomnia. Symptoms can be the direct result of lysis of red blood cells (e.g., hemoglobinuria, anemia, fatigue, low red blood cell count, etc.) or the symptoms can result from low nitric oxide (NO) levels in the patient's bloodstream (e.g., abdominal pain, erectile dysfunction, dysphagia, thrombosis, etc.). It has recently been reported that almost all patients with greater than 40% PNH type III granulocyte clone have thrombosis, abdominal pain, erectile dysfunction and dysphagia, indicating a high hemolytic rate (see Moyo et al., British J. Haematol. 126:133-138 (2004)).

In particularly useful embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a platelet count in excess of 40,000 per microliter (a hypoplastic patient), preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient where the proportion of PNH type III red blood cells of the subject's total red blood cell content is

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greater than 10%, preferably greater than 25%, most preferably in excess of 50%. In yet other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a reticulocyte count in excess of 80 x 10^9 per liter, more preferably in excess of 120 x 10^9 per liter, most preferably in excess of 150 x 10^9 per liter. Patients in the most preferable ranges recited above have active bone marrow and will produce adequate numbers of red blood cells. While in a patient afflicted with PNH or other hemolytic disease the red blood cells may be defective in one or more ways (e.g., GPI deficient), the present methods are particularly useful in protecting such cells from lysis resulting from complement activation. Thus, patients within the preferred ranges benefit most from the present methods.

In one aspect, a method of reducing fatigue is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing fatigue means the duration of time a person suffers from fatigue is reduced by about 25% or more. Fatigue is a symptom believed to be associated with intravascular hemolysis as the fatigue relents when hemoglobinuria resolves even in the presence of anemia. By reducing the lysis of red blood cells, the present methods reduce fatigue. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In another aspect, a method of reducing abdominal pain is contemplated, the method including the step of administering to a subject

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having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing abdominal pain means the duration of time a person suffers from abdominal pain is reduced by about 25% or more. Abdominal pain is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and intestinal dystonia and spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing abdominal pain. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In another aspect, a method of reducing dysphagia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing dysphagia means the duration of time a person has dysphagia attacks is reduced by about 25% or more. Dysphagia is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and esophageal spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing dysphagia. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

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In yet another aspect, a method of reducing erectile dysfunction is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing erectile dysfunction means the duration of time a person suffers from erectile dysfunction is reduced by about 25% or more. Erectile dysfunction is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing erectile dysfunction. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In yet another aspect, a method of reducing hemoglobinuria is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing hemoglobinuria means a reduction in the number of times a person has red, brown, or darker urine, wherein the reduction is typically about 25% or more. Hemoglobinuria is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream and urine thereby reducing hemoglobinuria. Quite surprisingly, the reduction in hemoglobinuria

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occurs rapidly. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In still another aspect, a method of reducing thrombosis is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing thrombosis means the duration of time a person has thrombosis attacks is reduced by about 25% or more. Thrombosis is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis and/or the lack of CD59 on the surface of platelets resulting in terminal complement mediated activation of the platelet. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, blockade of complement will prevent terminal complement-mediated activation of platelets and thrombosis. C5a will also be inhibited by this method which can induce platelet aggregation through C5a receptors on platelets and endothelial cells.

Thrombosis is thought to be multi-factorial in etiology including NO scavenging by free hemoglobin, the absence of terminal complement inhibition on the surface of circulating platelets and changes in the endothelium surface by cell free heme. The intravascular release of free hemoglobin may directly contribute to small vessel thrombosis. NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated

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platelets and inhibit platelet adhesion. Conversely, NO scavenging by hemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation. PNH platelets also lack the terminal complement inhibitor CD59 and multiple studies have shown that deposition of terminal complement (C5b-9) on platelets causes membrane vesiculation and the generation of microvesicles. The microvesicles act as a site for the generation of the clotting components factor Va, Xa or the prothrombinase complex. It is thought that these particles may also contribute to the genesis of thrombosis in PNH. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, inhibiting complement at C5 will prevent C5b-9 and C5a mediated activation of platelets and/or endothelial cells.

In particularly useful embodiments, the present methods reduce thrombosis, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods reduce thrombosis in patients where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods reduce transfusion thrombosis in patients having a reticulocyte count in excess of 80 x 10^9 per liter, more preferably in excess of 120 x 10^9 per liter, most preferably in excess of 150 x 10^9 per liter.

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In still another aspect, a method of reducing anemia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing anemia means the duration of time a person has anemia is reduced by about 25% or more. Anemia in hemolytic diseases results from the blood's reduced capacity to carry oxygen due to the loss of red blood cell mass. By reducing the lysis of red blood cells, the present methods assist red blood cell levels to increase thereby reducing anemia.

In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. By increasing the patient's RBC count, fatigue, anemia and the patient's need for blood transfusions is reduced. The reduction in transfusions can be in frequency of transfusions, amount of blood units transfused, or both.

The method of increasing red blood cell count in a patient afflicted with a hemolytic disease includes the step of administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. In particularly useful embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease where the proportion of PNH type III

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red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. In some embodiments, the methods of the present disclosure may result in a decrease in the frequency of transfusions by about 50%, typically a decrease in the frequency of transfusions by about 70%, more typically a decrease in the frequency of transfusions by about 90%.

In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. As those skilled in the art will appreciate, the normal life cycle for a red blood cell is about 120 days. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells. It has unexpectedly been found that in some patients transfusion-independence can be maintained for twelve months or more, in some cases more than two years, long beyond the 120 day life cycle of red blood cells. In particularly useful embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted

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with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter.

Methods of increasing the nitric oxide (NO) levels in a patient having PNH or some other hemolytic disease are also within the scope of the present disclosure. These methods of increasing NO levels include the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Low NO levels arise in patients afflicted with PNH or other hemolytic diseases as a result of scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO. In particularly useful embodiments, NO homeostasis is

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restored as evidenced by a resolution of symptoms attributable to NO deficiencies.

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 transfusions within twelve months. The median number of transfusions within the patient pool was nine in the previous twelve months. The median number of transfusion units used in the previous twelve months was twenty-two for the patient pool.

Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis. The first twelve weeks of the study constituted the pilot study. Following completion of the initial acute phase twelve week study, all patients participated in an extension study conducted to a total of 64 weeks. Ten of the eleven patients participated in an extension study conducted to a total of two years.

The effect of anti-C5 antibody treatments on PNH type III red blood cells ("RBCs") was tested. "PNH Type" refers to the density of GPI-anchored proteins expressed on the cell surface. Type I is normal expression, Type II is intermediate expression, and Type III has no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells is determined by flow

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cytometry in the manner described in Richards, et al., *Clin. Appl. Immunol. Rev.*, vol. 1, pages 315-330, 2001. As compared to pre-therapy conditions, PNH Type III red blood cells increased more than 50% during the extension study. The increase from a pre-study mean value of 36.7% of all red blood cells to a 64 week mean value of 58.4% of Type III red blood cells indicated that hemolysis had decreased sharply. See Table 1, below. Eculizumab therapy protected PNH type III RBCs from complement–mediated lysis, prolonging the cells survival. This protection of the PNH–affected cells reduced the need for transfusions, paroxysms and overall hemolysis in all patients in the trial.

Table 1
PNH Cell Populations
Pre- and Post- Eculizumab Treatment in All Patients

	Proportion of PNH Cells (%)			
PNH Cell Type	baseline	12 weeks	64 weeks	p-value[a]
Type III RBCs	36.7 +/- 5.9	59.2 +/- 8.0	58.4 +/- 8.5	0.005
Type II RBCs	5.3 +/- 1.4	7.5 +/- 2.1	13.2 +/- 2.4	0.013
Type III WBCs	92.1 +/- 4.6	89.9 +/- 6.6	91.1 +/- 5.8	N.S.
Type III Platelets	92.4 +/- 2.4	93.3 +/- 2.8	92.8+/- 2.6	N.S.

[a]comparison of mean change from baseline to 64 weeks

During the course of the two year extension study, it was found that PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9% (p=0.001) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% (p=0.01). There was no concomitant change in

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the proportion of PNH neutrophils in any of the patients during eculizumab therapy, indicating that the increase in the proportion of PNH red cells was due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) themselves.

The effect of anti-C5 antibody treatments on lactate dehydrogenase levels ("LDH") was measured on all eleven patients. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. The LDH levels were decreased by greater than 80% as compared to pre-treatment levels. The LDH levels were lowered from a pre-study mean value of 3111 U/L to a mean value of 594 U/L during the pilot study and a mean value of 622 U/L after 64 weeks ($p = 0.002$ for 64 week comparison; see Figures 1A and 1B).

Similarly, aspartate aminotransferase (AST) levels, another marker of red blood cell hemolysis, decreased from a mean baseline value of 76 IU/L to 26 IU/L and 30 IU/L during the 12 and 64 weeks of treatment, respectively ($p=0.02$ for 64 week comparison). Levels of haptoglobin, hemoglobin and bilirubin, and numbers of reticulocytes, did not change significantly from prestudy values during the 64 week treatment period.

Paroxysm rates were measured and compared to pre-treatment levels. Paroxysm as used in this disclosure is defined as incidences of dark-colored urine with a colorimetric level of 6 of more on a scale of 1-10. Figure 2 shows the urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in patients with PNH before and during treatment. As compared to pre-treatment levels, the paroxysm percentage rate was reduced

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by 93% (see, Figure 3) from 3.0 paroxysms per patient per month before eculizumab treatment to 0.1 paroxysms per patient per month during the initial 12 weeks and 0.2 paroxysms per patient per month during the 64 week treatment (Figure 3 ($p < 0.001$)).

Serum hemolytic activity in nine of the eleven patients was completely blocked throughout the 64 week treatment period with trough levels of eculizumab at equilibrium ranging from approximately 35 µg/ml to 350 µg/ml. During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14 day dosing interval, a pattern that was repeated between multiple doses. In one of the patients, as seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with the return of serum hemolytic activity. At the next dose, symptoms resolved (Figure 5) and reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days resulted in a regain of complement control which was maintained over the extension study to 64 weeks in both patients. This patient showed a 24 hour resolution of dysphagia and hemoglobinuria. A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab above 35 µg/ml and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients.

The patients' need for transfusions was also reduced by the treatment with eculizumab. Figure 6a compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5

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antibody for cytopenic patients, while Figure 6b compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for non-cytopenic patients. A significant reduction in the need for transfusion was also noted in the entire group (mean transfusion rates decreased from 2.1 units per patient per month during a 1 year period prior to treatment to 0.6 units per patient per month during the initial 12 weeks and 0.5 units per patient per month during the combined 64 week treatment period), with non-cytopenic patients benefiting the most. In fact, four of the non-thrombocytopenic patients with normal platelet counts (≥150,000 per microliter) became transfusion-independent during the 64 week treatment.

The effect of eculizumab administered in combination with erythropoietin (EPO) was also evaluated in a thrombocytopenic patient. EPO (NeoRecormon®, Roche Pharmaceuticals, Basel, Switzerland) was administered in an amount of 18,000 I.U. three times per week beginning in week 23 of the study. As shown in Figure 7, the frequency of transfusions required for this patient was significantly reduced, and soon halted.

For the two year extension study, 10 of the 11 patients from the initial 3 month study continued to receive 900 mg of eculizumab every other week. (One patient discontinued eculizumab therapy after 23 months.) Six of the 11 patients had normal platelets (no clinical evidence of marrow failure) whereas 5 of the 11 had low platelets. For the patient who discontinued eculizumab therapy after 23 months, intravascular hemolysis was successfully controlled by eculizumab, but the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below $30x10^9/l$, suggesting

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that the ongoing transfusions were likely a result of the underlying bone marrow failure.

Results of the two year extension study also demonstrated that there was a statistically significant decrease in transfusion requirements for the patients. Three patients remained transfusion independent during the entire two year treatment period, and four cytopenic patients became transfusion independent, three following treatment with EPO (NeoRecormon®). The reduction in transfusion requirements was found to be most pronounced in patients with a good marrow reserve.

Pharmacodynamic levels were measured and recorded according to eculizumab doses. The pharmacodynamic analysis of eculizumab was determined by measuring the capacity of patient serum samples to lyse chicken erythrocytes in a standard total human serum complement hemolytic assay. Briefly, patient samples or human control serum (Quidel, San Diego CA) was diluted to 40% vol/vol with gelatin veronal-buffered saline (GVB2+, Advanced Research technologies, San Diego, CA) and added in triplicate to a 96-well plate such that the final concentrations of serum in each well was 20%. The plate was then incubated at room temperature while chicken erythrocytes (Lampire Biologics, Malvern, PA) were washed. The chicken erythrocytes were sensitized by the addition of anti-chicken red blood cell polyclonal antibody (0.1% vol/vol). The cells were then washed and resuspended in GVB2+ buffer. Chicken erythrocytes (2.5 $x10^6$ cells/30 μL) were added to the plate containing human control serum or patient samples and incubated at 37°C for 30 min. Each plate contained six additional wells of identically prepared chicken erythrocytes of which four wells were incubated

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with 20% serum containing 2 mM EDTA as the blank and two wells were incubated with GVB2+ buffer alone as a negative control for spontaneous hemolysis. The plate was then centrifuged and the supernatant transferred to a new flat bottom 96-well plate. Hemoglobin release was determined at OD 415 nm using a microplate reader. The percent hemolysis was determined using the following formula:

$$\text{Percent Hemolysis} = 100 \times \frac{\text{(OD patient sample-OD blank)}}{\text{(OD human serum control-OD blank)}}$$

The graph of the pharmacodynamics (Figure 8), the study of the physiological effects, shows the percentage of serum hemolytic activity (i.e. the percentage of cell lysis) over time. Cell lysis was dramatically reduced in the majority of the patients to below 20% of normal serum complement activity while under eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, Figure 4).

Improvement of quality of life issues was also evaluated using the European Organization for Research and Treatment of Cancer Core (http://www.eortc.be) questionnaires ("EORTC QLC-C30"). Each of the participating patients completed the QLC-30 questionnaire before and during the eculizumab therapy. Overall improvements were observed in global health status, physical functioning, role functioning, emotional functioning, cognitive functioning, fatigue, pain, dyspnea and insomnia. (See Figure 9).

Patients in the two year study experienced a reduction in adverse symptoms associated with PNH. For example, as set forth in Figure 10, there was a demonstrated decrease of abdominal pain, dysphagia, and erectile

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dysfunction after administration of eculizumab in those patients reporting those symptoms before administration of eculizumab.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention.

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WHAT IS CLAIMED IS:

1. A method of treating a hemolytic disease in a subject comprising administering an anti-C5 antibody to a subject having a hemolytic disease, wherein there is a reduction in hemoglobinuria in the subject.

2. A method as in claim 1 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

3. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

4. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

5. A method as in claim 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

6. A method as in claim 1 wherein the subject's platelet count is greater than 40,000 per microliter.

7. A method as in claim 1 wherein the subject's platelet count is greater than 75,000 per microliter.

8. A method as in claim 1 wherein the subject's platelet count is greater than 150,000 per microliter.

9. A method as in claim 1 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

10. A method as in claim 1 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

11. A method as in claim 1 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

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12. A method as in claim 1 wherein said subject has a greater than 40% PNH type III granulocyte clone.

13. A method as in claim 1 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

14. A method of treating a hemolytic disease in a subject comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein there is a reduction in hemoglobinuria in the subject.

15. A method as in claim 14 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

16. A method as in claim 14 wherein said subject has a greater than 40% PNH type III granulocyte clone.

17. A method as in claim 14 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

18. A method of restoring nitric oxide (NO) homeostasis comprising administering an anti-C5 antibody to a subject having a hemolytic disease.

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19. A method as in claim 18 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

20. A method as in claim 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

21. A method as in claim 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

22. A method as in claim 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

23. A method as in claim 18 wherein the subject's platelet count is greater than 40,000 per microliter.

24. A method as in claim 18 wherein the subject's platelet count is greater than 75,000 per microliter.

25. A method as in claim 18 wherein the subject's platelet count is greater than 150,000 per microliter.

26. A method as in claim 18 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

27. A method as in claim 18 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

28. A method as in claim 18 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

29. A method as in claim 18 wherein said subject has a greater than 40% PNH type III granulocyte clone.

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30. A method as in claim 18 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

31. A method of restoring nitric oxide (NO) homeostasis comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

32. A method as in claim 31 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

33. A method as in claim 31 wherein said subject has a greater than 40% PNH type III granulocyte clone.

34. A method as in claim 31 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

35. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering an anti-C5 antibody, wherein hemolysis is reduced as evidenced by a greater than 50% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

36. A method as in claim 35 wherein hemolysis is reduced as evidenced by a greater than 65% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

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37. A method as in claim 35 wherein hemolysis is reduced as evidenced by a greater than 80% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

38. A method as in claim 35 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

39. A method as in claim 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

40. A method as in claim 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

41. A method as in claim 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

42. A method as in claim 35 wherein the subject's platelet count is greater than 40,000 per microliter.

43. A method as in claim 35 wherein the subject's platelet count is greater than 75,000 per microliter.

44. A method as in claim 35 wherein the subject's platelet count is greater than 150,000 per microliter.

45. A method as in claim 35 wherein the subject's reticulocyte count is greater than 80×10^9 per liter.

46. A method as in claim 35 wherein the subject's reticulocyte count is greater than 120×10^9 per liter.

47. A method as in claim 35 wherein the subject's reticulocyte count is greater than 150×10^9 per liter.

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48. A method as in claim 35 wherein said subject has a greater than 40% PNH type III granulocyte clone.

49. A method as in claim 35 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

50. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein hemolysis is reduced as evidenced by a greater than 50% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

51. A method as in claim 50 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

52. A method as in claim 50 wherein said subject has a greater than 40% PNH type III granulocyte clone.

53. A method as in claim 50 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

54. A method of reducing hemoglobinuria in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement

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components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

55. A method as in claim 54 wherein the step of administering comprises administering an anti-C5 antibody.

56. A method as in claim 54 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

57. A method as in claim 54 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

58. A method as in claim 54 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

59. A method as in claim 54 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

60. A method as in claim 54 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

61. A method as in claim 54 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

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62. A method as in claim 54 wherein the subject's platelet count is greater than 40,000 per microliter.

63. A method as in claim 54 wherein the subject's platelet count is greater than 75,000 per microliter.

64. A method as in claim 54 wherein the subject's platelet count is greater than 150,000 per microliter.

65. A method as in claim 54 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

66. A method as in claim 54 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

67. A method as in claim 54 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

68. A method as in claim 54 wherein said subject has a greater than 40% PNH type III granulocyte clone.

69. A method as in claim 54 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

70. A method of reducing dysphagia in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components and compounds which block the generation of one or more complement components.

71. A method as in claim 70 wherein the step of administering comprises administering an anti-C5 antibody.

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72. A method as in claim 70 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

73. A method as in claim 70 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

74. A method as in claim 70 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

75. A method as in claim 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

76. A method as in claim 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

77. A method as in claim 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

78. A method as in claim 70 wherein the subject's platelet count is greater than 40,000 per microliter.

79. A method as in claim 70 wherein the subject's platelet count is greater than 75,000 per microliter.

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80. A method as in claim 70 wherein the subject's platelet count is greater than 150,000 per microliter.

81. A method as in claim 70 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

82. A method as in claim 70 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

83. A method as in claim 70 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

84. A method as in claim 70 wherein said subject has a greater than 40% PNH type III granulocyte clone.

85. A method as in claim 70 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

86. A method of reducing erectile dysfunction in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components and compounds which block the generation of one or more complement components.

87. A method as in claim 86 wherein the step of administering comprises administering an anti-C5 antibody.

88. A method as in claim 86 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

89. A method as in claim 86 wherein the step of administering comprises administering a compound selected from the group consisting of

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